

1/28/00
TRANSMITTAL LETTER

To the Commissioner of Patents and Trademarks:

003/115/SAP RMD96-10

Client/Matter

Transmitted herewith for filing is the X Utility Design Plant patent application of :

Inventor(s): Connie S. Schmaljohn and J. W. Hooper

Title of invention: DNA Vaccines Against Hantavirus Infections

Enclosed are the following items:

- X Duplicate copies of this Patent Application Transmittal form.
7 Sheet(s) of (X) informal () formal drawings.
41 Page(s) of specification.
4 Page(s) of claims.
1 Page(s) of abstract.
 Declaration () signed () unsigned.
 A Power of Attorney executed by the () inventors () Assignee.
 An assignment transmittal letter.
 A verified statement to establish Small Entity Status under 37 CFR 1.9 and 37 CFR 1.27.
 An information Disclosure Statement.
 A list of references (Form-1449).
 Copies of references.
 Preliminary amendment.
10 Sheets Sequence Listing
X Postcard.
 Other (specify) COMPUTER READABLE FORM OF SEQUENCE LISTING

CLAIMS AS FILED

BASIC FILING FEE

<u>X</u> UTILITY	SMALL ENTITY	OR	OTHER THAN SMALL ENTITY	
	\$ 380		\$ 760	\$ <u>760</u>
<u> </u> DESIGN	\$ 165	OR	\$ 330	\$ <u> </u>
<u> </u> PLANT	\$ 270	OR	\$ 540	\$ <u> </u>

TOTAL CLAIMS 27 - 20 = 7 X \$ 9 OR \$ 18 = \$ 126

INDEPENDENT CLAIMS 4 - 3 = 1 X \$ 39 OR \$ 78 = \$ 312

MULTIPLE DEPENDENT CLAIMS PRESENT + \$ 130 OR \$ 260 = \$

RECORDING FEE FOR ASSIGNMENT (\$ 40.00/EA.) \$

TOTAL FILING FEE: \$ 1,198.00

The Commissioner is hereby authorized to charge and credit Deposit Account No. 21-0380 as described below:

- X Charge the total filing fee of \$ 1,198.00
X Charge any additional filing fees as required under 37 CFR 1.16 and 1.17.
X Credit any overpayment.
X Charge the issue fee required under 37 CFR 1.18 at the time of mailing of the Notice of Allowance, pursuant to 37 CFR 1.311(b).

Claim for Priority Under 35 U.S.C., Section 119(e):

(X) The benefit under 35 U.S.C., Section 119(e) is hereby claimed from the following United States application:
Provisional Application Serial No. 60/117,680 filed 29 January 1999 still pending.

15598 U.S. PRO
09/491974
01/27/00

Please address all correspondence in connection with this application to:

Sana A. Pratt, Ph.D.
Pratt & Associates, Inc.
10821 Hillbrooke Lane
Potomac, Maryland 20854

By: *S. A. Pratt*, Reg. No. 39,441 Date: 27 January 2000
for Charles H. Harris, Reg. No. 34,616

I hereby certify that this correspondence is being deposited with the United States Postal Service in an Express Mail envelope, Mailing Label NO. EL437490864US, addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231, on the date of 27 January 2000.

Respectfully submitted,

S. A. Pratt
Sana A. Pratt, Reg. No. 39,441
Tel.: 301-294-9171

SAP 10/96

TITLE OF THE INVENTION**DNA Vaccines Against Hantavirus Infections**

by

5 Connie S. Schmaljohn
 J. W. Hooper

INTRODUCTION

10 Currently, there are four known hantaviruses
 associated with hemorrhagic fever with renal syndrome
 (HFRS): Hantaan virus (HTNV), Dobrava-Belgrade virus
 (DOBV), Puumala virus (PUUV), and Seoul virus (SEOV).
 Because distinct hantaviruses are usually carried by
 only one principal rodent host species, their
15 distribution is generally limited to the range of that
 host (reviewed in Schmaljohn and Hjelle, 1997, *Emerg.*
 Infect. Dis. 3, 95-104). HTNV, carried by *Apodemus*
 agrarius, is found in Asia; DOBV, carried by *Apodemus*
 flavicollis, and PUUV, carried by *Clethrionomys*
20 *glareolus*, are found in Europe. SEOV is more widely
 disseminated than any other recognized hantavirus
 because its host, the common urban rat (*Rattus*
 norvegicus), is found throughout the world.

 Viruses in the *Hantavirus* genus (family
25 *Bunyaviridae*) are enveloped and contain a genome
 comprised of three single-stranded RNA segments
 designated large (L), medium (M), and small (S) based
 on size (reviewed in Schmaljohn, 1996, In The
 Bunyaviridae Ed. R. M. Elliott. New York, Plenum Press
30 p. 63-90). The hantavirus L segment encodes the RNA
 dependent RNA polymerase, M encodes two envelope
 glycoproteins (G1 and G2), and S encodes the
 nucleocapsid protein (N).

A number of inactivated HFRS vaccines derived from cell culture or rodent brain were developed and tested in Asia (Lee et al., 1990, *Arch. Virol.*, Suppl. 1, 35-47; Song et al., 1992, *Vaccine* 10, 214-216; Lu et al., 1996, *J. Med. Virol.* 49, 333-335). Other vaccine approaches involving recombinant DNA technology include: vaccinia-vectored vaccines (Schmaljohn et al. 1990, *J. Virol.* 64, 3162-3170; Schmaljohn et al. 1992, *Vaccine* 10, 10-13; Xu et al. 1992, *Am. J. Trop. Med. Hyg.* 47, 397-404), protein subunit vaccines expressed in bacteria or insect cells (Schmaljohn et al. 1990, *supra*; Yoshimatsu et al., 1993, *Arch. Virol.* 130, 365-376; Lundkvist et al., 1996, *Virology* 216, 397-406), and a hepatitis core antigen-based recombinant vaccine (Ulrich et al., 1998, *Vaccine* 16, 272-280).

Hantavirus G1, G2, and N proteins are known to elicit immune responses; however, the relative importance these proteins play in providing protection remains an area of investigation. Vaccination with vaccinia recombinants expressing the M segment of either HTNV or SEOV elicited neutralizing antibodies and protected rodents against infection with both HTNV and SEOV, suggesting that an immune response to G1-G2 alone can confer protection (Schmaljohn et al. 1990, *supra*; Xu et al. 1992, *supra*; Chu et al. 1995, *J. Virol.* 69, 6417-6423). Similarly, vaccination with G1-G2 protein expressed in insect cells (baculovirus recombinant virus system) elicited neutralizing antibodies and protected hamsters from infection with HTNV (Schmaljohn et al. 1990, *supra*). In both the vaccinia and baculovirus systems, vaccination with G1-G2 provided more complete protection than G1 or G2 alone (Schmaljohn et al. 1990, *supra*). Neutralizing antibody responses to G1-G2 in the aforementioned

vaccine studies correlated with protection, suggesting that neutralizing antibodies play an important role in preventing hantavirus infection. Passive transfer of neutralizing monoclonal antibodies (Mabs) specific to either G1 or G2 protected hamsters against HTNV infection (Schmaljohn et al., 1990, supra; Arikawa et al., 1992, *J. Gen. Virol.* 70, 615-624), supporting the idea that neutralizing antibodies alone can confer protection. The N protein also plays a role in protecting against hantavirus infection. Vaccination with N expressed in bacteria, insect cells, or as chimeric hepatitis B virus (HBV) core particles protected rodents from hantavirus infection (Schmaljohn et al., 1990, supra; Yoshimatsu et al. 1993, supra; Lundkvist et al., 1996, supra; Ulrich et al., 1998, supra). Vaccination with vaccinia recombinants expressing the S segment were less conclusive. A construct expressing the HTNV S segment did not protect hamsters from HTNV infection, possibly due to low N expression levels (Schmaljohn et al. 1990, supra); and a construct expressing the S segment of SEOV protected three of four gerbils from SEOV infection (Xu et al. 1992, supra).

Recombinant vaccinia virus vaccines and vaccinia-based vaccines present disadvantages including the potential for disseminated infection, especially in immunocompromised individuals, since the vaccines consist of live virus. Also, vaccination with these viruses can result in a lesion (pock) that contains infectious virus. Virus from these lesions can be inadvertently spread to other sites (e.g., eyes) or to other individuals. Other drawbacks of vaccinia-based vaccines include discomfort due to swollen lymphnodes and scarring at the site of inoculation.

DNA vaccination mimicks the de novo antigen production and MHC class I-restricted antigen presentation obtainable with live vaccines, without the risks of pathogenic infection.

- 5 This vaccine approach is advantageous over subunit vaccine which do not elicit a cytotoxic response necessary to prevent the establishment of infection or disease. Also, this DNA vaccine approach allows delivery to mucosal tissues which may aid in
10 conferring resistance to viral introduction since entry of the virus may be through mucosal tissues.

SUMMARY OF THE INVENTION

- In this report, we describe a new recombinant DNA vaccine approach that involves vaccination with naked
15 DNA expressing individual hantavirus genome segment cDNAs. Naked DNA vaccination involves delivery of plasmid DNA constructs with a gene(s) of interest into the tissue of the vaccinee (reviewed in Robinson and Torres, 1997, *Semin. Immunol.* 9, 271-283; and
20 Gregoriadis, 1998, *Pharm. Res.* 15, 661-670). The gene(s) of interest, in our case, a hantavirus genome segment, is controlled by a mammalian or virus promoter (e.g., the cytomegalovirus immediate early promoter) that facilitates expression of the naked DNA
25 gene product(s) within the vaccinee's cells. This intracellular expression can elicit both humoral and cell-mediated immune responses (Robinson and Torres, 1997, *supra*; and Gregoriadis, 1998, *supra*). Methods of DNA delivery include needle inoculation, oral or
30 pulmonary delivery, and inoculation by particle bombardment (i.e., gene gun). DNA vaccination by each of these methods elicits protective immunity against many different pathogens including numerous viruses (Robinson and Torres, 1997, *supra*; and Gregoriadis,
35 1998, *supra*). However, neither an immune response

against hantaviruses nor protection against hantavirus infection have been demonstrated using a DNA vaccine.

In this report, we demonstrate that naked DNA vaccination with the SEOV M or S genome segment elicits SEOV-specific antibody responses in rodents. More importantly, we demonstrate that DNA vaccination with the SEOV M segment elicits neutralizing antibodies and protects hamsters against SEOV infection.

Therefore, it is one object of the present invention to provide a hantavirus DNA vaccine comprising a hantavirus genome segment. More specifically, the present invention relates to an SEOV hantavirus DNA vaccine comprising the SEOV M genome segment specified in SEQ ID NO:1 or comprising the SEOV S segment specified in SEQ ID NO:2.

It is another object of the present invention to provide a method for eliciting in a subject an immune response against hantavirus, the method comprising administering to a subject a DNA fragment comprising a genome segment of hantavirus. More specifically, the present invention relates to a method for eliciting an immune response against SEOV hantavirus by providing the M or S genome segment of SEOV. Cross protection against other hantaviruses such as Hantaan virus and Dobrava are also an object of this invention.

In one aspect of the invention, the DNA vaccine is delivered by coating a small carrier particle with the DNA vaccine and delivering the DNA-coated particle into an animal's epidermal tissue via particle bombardment. This method may be adapted for delivery to either epidermal or mucosal tissue, or delivery into peripheral blood cells, and thus may be

used to induce humoral, cell-mediated, and secretory immune responses in the vaccinated individual.

The DNA vaccine according to the present invention is inherently safe, is not painful to administer, and should not result in adverse side effects to the vaccinated individual. In addition, the invention does not require growth or use of hantavirus, which may be spread by aerosol transmission.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and accompanying drawings where:

FIG. 1. SEOV naked DNA expression constructs.

The SEOV M or S genome segment was amplified by RT-PCR and cloned into NotI and BamHI sites of pWRG7077 (PowderJect Vaccines, Inc., Madison, Wisconsin, described in Schmaljohn et al., 1997, supra). Characteristics of pWRG7077 are similar to those of pWRG1602 described previously (Dimmock, N. J., 1995, *Med. Virol.* 5: 165) and include a human cytomegalovirus early promoter (CMV IE promoter) and intron A, a bovine growth hormone transcription terminator and polyadenylation signal (BGH pA), and a kanamycin resistance gene. CMV IE promoter: cytomegalovirus immediate early promoter and intron A, BGH pA: Bovine growth hormone polyadenylation signal, KAN: kanamycin antibiotic resistance gene.

FIG. 2 A and B. Transient expression from pWRG/SEO-M (SEQ ID NO:3) and pWRG/SEO-S (SEQ ID NO:4). A) IFAT were performed on COS cell monolayers transfected with the SEOV M or S constructs. pWRG/7077-transfected monolayers served as negative

controls. Anti-SEOV polyclonal rabbit antisera was used as the primary antibody. B) COS cells were transfected with the indicated plasmid and radiolabeled expression products were

- 5 immunoprecipitated with anti-SEOV polyclonal rabbit serum. Molecular size markers (MW) in kDa are shown on the left, and the position of the SEOV G1, G2, and N proteins are shown on the right.

FIG. 3A and 3B. Antibody responses (ELISA) of mice vaccinated with SEOV naked DNA by gene gun or intramuscular needle inoculation. The antibody responses to vaccination with pWRG/SEO-M (panel A) or pWRG/SEO-S (panel B) by gene gun or intramuscular needle inoculation were evaluated by ELISA. Mice vaccinated with negative control plasmid pWRG7077 were evaluated in both ELISAs. Prebleed sera and sera collected 4 weeks after the first vaccination (1 vacc), 4 weeks after the second vaccination (2 vacc), 3 weeks after the final vaccination (3 vacc) were all tested in a single assay. Symbols represent the average values of individual mice serum samples assayed in duplicate.

FIG. 4. Neutralizing antibody responses of mice vaccinated with SEOV naked DNA. PRNT were performed on mice sera collected 3 weeks after the third vaccination by either gene gun or intramuscular needle injection (needle). Bars represent sera from individual mice vaccinated with the indicated immunogen. Sera were heat inactivated (56°C, 30 min) and the assay was performed in the presence of 5% guinea pig complement. PRNT_{50%} titers are expressed as reciprocal of the highest serum dilution resulting in a 50% reduction in plaque number.

FIG. 5. Vaccination with pWRG/SEO-M cross-protects against infection with Hantaan virus. Groups

of 4-5 hamsters were vaccinated with either pWRG/SEO-M or negative control plasmid (pWRG7077) as described in Methods below. Three weeks after the final vaccination, prechallenge serum samples were obtained and the hamsters were challenged with 1,000 PFU of Hantaan virus. Twenty-eight days after challenge postchallenge serum samples were obtained. Pre and postchallenge serum samples were evaluated by anti-N ELISA to detect antibody to nucleocapsid, and by a Hantaan plaque reduction neutralization test (PRNT) to detect Hantaan neutralizing antibodies. ELISA titers represent the lowest reciprocal dilution that resulted in an O.D. value that was greater than the background O.D. plus three standard deviations. PRNT50% titers represent the lowest reciprocal dilution that resulted in a 50% reduction in plaque number in the absence of serum.

FIG. 6. Vaccination with pWRG/SEO-M cross-protects against infection with Dobrava virus. Groups of 5 hamsters were vaccinated with either pWRG/SEO-M or negative control plasmid (pWRG7077) as described in Methods. Three weeks after the final vaccination, prechallenge serum samples were obtained and the hamsters were challenged with 1,000 PFU of Dobrava virus. Twenty-eight days after challenge postchallenge serum samples were obtained. Pre and postchallenge serum samples were evaluated by anti-N ELISA to detect antibody to nucleocapsid, and by a Dobrava plaque reduction neutralization test (PRNT) to detect Dobrava neutralizing antibodies. ELISA titers represent the lowest reciprocal dilution that resulted in an O.D. value that was greater than the background O.D. plus three standard deviations. PRNT50% titers represent the lowest reciprocal dilution that resulted in a 50% reduction in plaque number in the absence of

serum. The Seoul neutralizing prechallenge antibody titers (elicited by the vaccine) were also measured. The Seoul virus PRNT80% values are shown as empty circles.

- 5 **FIG. 7.** Vaccination with pWRG/SEO-M fails to cross-protect against infection with Puumala virus. Groups of 5 hamsters were vaccinated with either pWRG/SEO-M or negative control plasmid (pWRG7077) as described in Methods. Three weeks after the final
- 10 vaccination, prechallenge serum samples were obtained and the hamsters were challenged with 1,000 PFU of Puumala virus. Twenty-eight days after challenge postchallenge serum samples were obtained. Pre and postchallenge serum samples were evaluated by anti-N
- 15 ELISA to detect antibody to nucleocapsid, and by a Puumala plaque reduction neutralization test (PRNT) to detect Puumala neutralizing antibodies. ELISA titers represent the lowest reciprocal dilution that resulted in an O.D. value that was greater than the background
- 20 O.D. plus three standard deviations. PRNT50% titers represent the lowest reciprocal dilution that resulted in a 50% reduction in plaque number in the absence of serum. The Seoul neutralizing prechallenge antibody titers (elicited by the vaccine) were also measured.
- 25 The Seoul virus PRNT80% values are shown as empty circles.

DETAILED DESCRIPTION

- In this application is described a composition and method for the vaccination of individuals against
- 30 hantavirus. The method comprises delivery of a DNA encoding a hantavirus antigen to cells of an individual such that the antigen is expressed in the cell and an immune response is induced in the individual.

DNA vaccination involves administering antigen-encoding polynucleotides *in vivo* to induce the production of a correctly folded antigen(s) within the target cells. The introduction of the DNA vaccine

15 will cause to be expressed within those cells the structural protein determinants associated with the pathogen protein or proteins. The processed structural proteins will be displayed on the cellular surface of the transfected cells in conjunction with

10 the Major Histocompatibility Complex (HMC) antigens of the normal cell. Even when cell-mediated immunity is not the primary means of preventing infection, it is likely important for resolving established infections. Furthermore, the structural proteins released by the

15 expressing transfected cells can also be picked up by antigen-presenting cells to trigger systemic humoral antibody responses.

In order to achieve the immune response sought, a DNA vaccine construct capable of causing transfected

20 cells of the vaccinated individual to express one or more major viral antigenic determinant is necessary. This can be done by identifying regions of the viral genome which code for viral glycoproteins or capsid components, and joining such coding sequences to

25 promoters capable of expressing the sequences in cells of the vaccinee. Alternatively, the viral genome itself, or parts of the genome, can be used.

In one embodiment, the present invention relates to a DNA or cDNA segment which encodes an antigen from

30 a hantavirus. By hantavirus is meant any of the Hemorrhagic fever with renal syndrome (HFRS) hantavirus such as Hantaan virus (HTNV), Dobrava-Belgrade virus (DOBV), Puumala virus (PUUV), and Seoul virus (SEOV), as well as hantavirus pulmonary syndrome

35 (HPS) hantaviruses such as Sin Nombre virus (SNV),

Black Creek Canal virus (BCCV), Bayou virus (BAYV), New York virus (NYV), Andes virus (ANDV), Laguna Negra virus (LNV), and any other hantavirus known to cause disease in humans.

- 5 More specifically, a hantavirus genome M segment encoding two envelope glycoproteins (G1 and G2) (SEQ ID NO:1), and a hantavirus genome S segment encoding nucleocapsid protein (SEQ ID NO:2) were deduced from the SEOV, strain SR-11 (Kitamura, T. et al. 1983, *Jpn. J. Med. Sci. Biol.* 36, 17-25) viral genome (Arikawa, J. et al. 1990, *Virology* 176, 114-125). The M segment (specified in SEQ ID NO:1), deposited in GenBank accession no. M34882, corresponds to Seoul (SR-11) M gene from nucleotide 2 to 3602. The S segment (SEQ ID NO:2), having Genbank accession no. M34881, corresponds to Seoul (SR-11) S gene from nucleotide 2 to 1699.

- DNA or polynucleotide sequences to which the invention also relates include fragments of the M or S gene segment from other Hantaviruses containing protective epitopes or antigenic determinants. Such epitopes, which may be conformational, may include sequences from G1 and/or G2 since monoclonal antibodies to both G1 and G2 have been shown to neutralize virus and protect rodents in passive protection experiments. Additionally, the amino terminus of N is highly immunogenic and others have shown that other methods of vaccination with the amino terminal region can confer protection (Lundvist, 1996, supra; Ulrich, 1998, supra).

- The derived sequence is not necessarily physically derived from the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:2, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or

transcription, which are based on the information provided by the sequence bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain reaction (PCR) assays for the detection of Hantavirus.

RT-PCR cloning of the M and S genome segments of SEOV, strain SR-11, was described previously (Arikawa et al., 1990, Virology 176, 114-125). A DNA fragment containing Seoul (SR-11) M gene from nucleotide 2 to 3602 was excised from YMIS/SR 11-M (Arikawa, 1990, supra) using Bgl II and Bam HI. This fragment was ligated into the Bam HI site of pWRG7077 resulting in pWRG/SEO-M (SEQ ID NO:3). pWRG/SEO-M contains additional nucleotides derived from cloning vector baculovirus transfer vector YMIS.

A DNA fragment containing Seoul (SR-11) S gene from nucleotide 2 to 1699 was excised from pBSKS+/SR11-S (Arikawa, 1990, supra) using EcoRI. This fragment was subjected to Klenow to blunt the restriction sites and then ligated into Klenow-blunted Not I site of pWRG7077 (provided by Powderject, Inc., Madison, Wisconsin) resulting in pWRG/SEO-S (SEQ ID NO:4). pWRG/SEO-S contains additional nucleotides derived from cloning vector pBSKS+.

It is understood in the art that certain changes to the nucleotide sequence employed in a genetic construct have little or no bearing on the proteins encoded by the construct, for example due to the degeneracy of the genetic code. Such changes result either from silent point mutations or point mutations

that encode different amino acids that do not appreciably alter the behavior of the encoded protein. It is also understood that portions of the coding region can be eliminated without affecting the ability of the construct to achieve the desired effect, namely induction of a protective immune response against hantavirus. It is further understood in the art that certain advantageous steps can be taken to increase the antigenicity of an encoded protein by modifying its amino acid composition. Such changes in amino acid composition can be introduced by modifying the genetic sequence encoding the protein. It is contemplated that all such modifications and variations of the M and S segments of hantavirus are equivalents within the scope of the present invention.

The DNA encoding the desired antigen can be introduced into the cell in any suitable form including, a linearized plasmid, a circular plasmid, a plasmid capable of replication, an episome, RNA, etc. Preferably, the gene is contained in a plasmid. In a particularly preferred embodiment, the plasmid is an expression vector. Individual expression vectors capable of expressing the genetic material can be produced using standard recombinant techniques. Please see e.g., Maniatis et al., 1985 Molecular Cloning: A Laboratory Manual or DNA Cloning, Vol. I and II (D. N. Glover, ed., 1985) for general cloning methods.

Therefore, in another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid such as pCRII (Invitrogen) or pJW4303 (Konishi, E. et al., 1992, *Virology* 188:714), or any expression vector such

as viral vectors e.g. adenovirus or Venezuelan equine encephalitis virus and others known in the art.

Preferably, a promoter sequence operable in the target cell is operably linked to the DNA sequence. Several

- 5 such promoters are known for mammalian systems which may be joined 5', or upstream, of the coding sequence for the encoded protein to be expressed. A suitable promoter is the human cytomegalovirus immediate early promoter. A downstream transcriptional terminator, or
10 polyadenylation sequence, such as the polyA addition sequence of the bovine growth hormone gene, may also be added 3' to the protein coding sequence.

- A suitable construct for use in the method of the present invention is pWRG7077 (4326 bp) (PowderJect
15 Vaccines, Inc., Madison, WI), Figure 1. pWRG7077 includes a human cytomegalovirus (hCMV) immediate early promoter (IE) and a bovine growth hormone polyA addition site. Between the promoter and the polyA addition site is Intron A, a sequence that naturally
20 occurs in conjunction with the hCMV IE promoter that has been demonstrated to increase transcription when present on an expression plasmid. Downstream from Intron A, and between Intron A and the polyA addition sequence, are unique cloning sites into which the
25 hantavirus M or S DNA can be cloned. Also provided on pWRG7077 is a gene that confers bacterial host-cell resistance to kanamycin. Any of the fragments that encode hantavirus G1 or G2 or nucleocapsid peptides can be cloned into one of the cloning sites in
30 pWRG7077, using methods known to the art.

- In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA construct. The host cell can be prokaryotic such as
35 *Bacillus* or *E. coli*, or eukaryotic such as

Saccharomyces or *Pichia*, or mammalian cells or insect cells. The vector containing the hantavirus sequence is expressed in the bacteria and the expressed product used for diagnostic procedures or as a vaccine.

- 5 Please see e.g., Maniatis et al., 1985 Molecular Cloning: A Laboratory Manual or DNA Cloning, Vol. I and II (D. N. Glover, ed., 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG
- 10 molecule, an adjuvant, a carrier, or an agent for aid in purification of hantavirus proteins or peptides. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an
- 15 expression system, the transformed or transfected cells can be used as a source of the protein or peptide encoded by the DNA. The DNA can be used as circular or linear, or linearized plasmid as long as the hantavirus sequences are operably linked to a
- 20 promoter which can be expressed in the transfected cell.

- In this application we describe the elicitation of protective immunity to hantaviruses by DNA vaccines. The DNA can be delivered by injection into
- 25 the tissue of the recipient, oral or pulmonary delivery and inoculation by particle bombardment (i.e., gene gun). Any of these methods can be used to deliver DNA as long as the DNA is expressed and the desired antigen is made in the cell. Two methods are
 - 30 exemplified in this application, both shown to be successful in eliciting a protective immune response in the vaccinee.

To deliver DNA vaccines by particle bombardment, we chose to use the *PowderJect-XR™* gene gun device

described in WO 95/19799, 27 July 1995. Other instruments are available and known to people in the art. This instrument, which delivers DNA-coated gold beads directly into epidermal cells by high-velocity particle bombardment, was shown to more efficiently induce both humoral and cell-mediated immune responses, with smaller quantities of DNA, than inoculation of the same DNAs by other parenteral routes (Eisenbraun, M. et al., 1993, *DNA Cell. Biol.* 12: 791; Fynan, E. F. et al., 1993, *Proc. Natl. Acad. Sci. USA* 90: 11478; Haynes, J. R. et al., 1994, *AIDS Res. Hum. Retroviruses* 10: Suppl. 2:S43; Pertmer, T. M. et al., 1995, *Vaccine* 13: 1427). Epidermal inoculation of the DNA candidate vaccines also offers the advantages of gene expression in an immunologically active tissue that is generally exfoliated within 15 to 30 days, and which is an important natural focus of viral replication after tick-bite (Bos, J. D., 1997, *Clin. Exp. Immunol.* 107 Suppl. 1:3; Labuda, M. et al., 1996, *Virology* 219:357; Rambukkana, A. et al., 1995, *Lab. Invest.* 73:521; Stingl, G., 1993, *Recent Results Cancer Res.* 128:45). Candidate vaccines include particles having M genome segments, including G1 and/or G2, from different HFRS viruses such as Seoul virus, Hantaan virus, Puumala virus, and Dobrava virus, as well as Hantavirus pulmonary syndrome virus such as Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus. The DNA segments from different viruses can be on different particles or on the same particle, whichever results in the desired immune response. In addition, the present invention relates to a vaccine comprising one or more DNAs from different hantaviruses. Such a vaccine is referred to as a multivalent vaccine. The

vaccine is designed to protect against pathologies resulting from exposure to one or several hantaviruses. The vaccine can also be combined with reagents which increase the antigenicity of the vaccine, or reduce its side effects.

The technique of accelerated particles gene delivery or particle bombardment is based on the coating of DNA to be delivered into cells onto extremely small carrier particles, which are designed to be small in relation to the cells sought to be transformed by the process. The DNA sequence containing the desired gene can be simply dried onto a small inert particle. The particle may be made of any inert material such as an inert metal (gold, silver, platinum, tungsten, etc.) or inert plastic (polystyrene, polypropylene, polycarbonate, etc.). Preferably, the particle is made of gold, platinum or tungsten. Most preferably, the particle is made of gold. Suitably, the particle is spherical and has a diameter of 0.5 to 5 microns, preferably 1 to 3 microns.

The DNA sequence containing the desired gene prepared in the form suitable for gene introduction can be simply dried onto naked gold or tungsten pellets. However, DNA molecules in such a form may have a relatively short period of stability and may tend to degrade rather rapidly due to chemical reactions with the metallic or oxide substrate of the particle itself. Thus, if the carrier particles are first coated with an encapsulating agent, the DNA strands have greatly improved stability and do not degrade significantly even over a time period of several weeks. A suitable encapsulating agent is polylysine (molecular weight 200,000) which can be applied to the carrier particles before the DNA

molecules are applied. Other encapsulating agents, polymeric or otherwise, may also be useful as similar encapsulating agents, including spermidine. The polylysine is applied to the particles by rinsing the gold particles in a solution of 0.02% polylysine and then air drying or heat drying the particles thus coated. Once the metallic particles coated with polylysine were properly dried, DNA strands are then loaded onto the particles.

The DNA is loaded onto the particles at a rate of between 0.5 and 30 micrograms of DNA per milligram of gold bead spheres. A preferable ratio of DNA to gold is 0.5-5.0 ug of DNA per milligram of gold. A sample procedure begins with gamma irradiated (preferably about 30 kGy) tefzel tubing. The gold is weighed out into a microfuge tube, spermidine (free base) at about 0.05 M is added and mixed, and then the DNA is added. A 10% CaCl solution is incubated along with the DNA for about 10 minutes to provide a fine calcium precipitate. The precipitate carries the DNA with it onto the beads. The tubes are microfuged and the pellet resuspended and washed in 100% ethanol and the final product resuspended in 100% ethanol at 0.0025mg/ml PVP. The gold with the DNA is then applied onto the tubing and dried.

The general approach of accelerated particle gene transfection technology is described in U.S. Patent No. 4,945,050 to Sanford. An instrument based on an improved variant of that approach is available commercially from PowderJect Vaccines, Inc., Madison Wisconsin, and is described in WO 95/19799. All documents cited herein *supra* and *infra* are hereby incorporated in their entirety by reference thereto. Briefly, the DNA-coated particles are deposited onto the interior surface of plastic tubing which is cut to

a suitable length to form sample cartridges. A sample cartridge is placed in the path of a compressed gas (e.g., helium at a pressure sufficient to dislodge the particles from the cartridge e.g., 350-400 psi). The particles are entrained in the gas stream and are delivered with sufficient force toward the target tissue to enter the cells of the tissue. Further details are available in the published apparatus application.

10 The coated carrier particles are physically accelerated toward the cells to be transformed such that the carrier particles lodge in the interior of the target cells. This technique can be used either with cells in vitro or in vivo. At some frequency, 15 the DNA which has been previously coated onto the carrier particles is expressed in the target cells. This gene expression technique has been demonstrated to work in prokaryotes and eukaryotes, from bacteria and yeasts to higher plants and animals. Thus, the 20 accelerated particle method provides a convenient methodology for delivering genes into the cells of a wide variety of tissue types, and offers the capability of delivering those genes to cells in situ and in vivo without any adverse impact or effect on 25 the treated individual. Therefore, the accelerated particle method is also preferred in that it allows a DNA vaccine capable of eliciting an immune response to be directed both to a particular tissue, and to a particular cell layer in a tissue, by varying the 30 delivery site and the force with which the particles are accelerated, respectively. This technique is thus particularly suited for delivery of genes for antigenic proteins into the epidermis.

A DNA vaccine can be delivered in a non-invasive 35 manner to a variety of susceptible tissue types in

order to achieve the desired antigenic response in the individual. Most advantageously, the genetic vaccine can be introduced into the epidermis. Such delivery, it has been found, will produce a systemic humoral
5 immune response.

To obtain additional effectiveness from this technique, it may also be desirable that the genes be delivered to a mucosal tissue surface, in order to ensure that mucosal, humoral and cellular immune
10 responses are produced in the vaccinated individual. There are a variety of suitable delivery sites available including any number of sites on the epidermis, peripheral blood cells, i.e. lymphocytes, which could be treated in vitro and placed back into
15 the individual, and a variety of oral, upper respiratory, and genital mucosal surfaces.

Gene gun-based DNA immunization achieves direct, intracellular delivery of DNA, elicits higher levels of protective immunity, and requires approximately
20 three orders of magnitude less DNA than methods employing standard inoculation.

Moreover, gene gun delivery allows for precise control over the level and form of antigen production in a given epidermal site because intracellular DNA
25 delivery can be controlled by systematically varying the number of particles delivered and the amount of DNA per particle. This precise control over the level and form of antigen production may allow for control over the nature of the resultant immune response.

30 The term transfected is used herein to refer to cells which have incorporated the delivered foreign DNA vaccine, whichever delivery technique is used.

It is herein disclosed that when inducing cellular, humoral, and protective immune responses
35 after DNA vaccination the preferred target cells are

epidermal cells, rather than cells of deeper skin layers such as the dermis. Epidermal cells are preferred recipients of DNA vaccines because they are the most accessible cells of the body and may, therefore, be immunized non-invasively. Secondly, in addition to eliciting a humoral immune response, DNA immunized epidermal cells also elicit a cytotoxic immune response that is stronger than that generated in sub-epidermal cells. Delivery to epidermis also has the advantages of being less invasive and delivering to cells which are ultimately sloughed by the body.

Although it can be desirable to induce an immune response by delivering genetic material to a target animal, merely demonstrating an immune response is not necessarily sufficient to confer protective advantage on the animal. What is important is to achieve a protective immune response that manifests itself in a clinical difference. That is, a method is effective only if it reduces the severity of the disease symptoms. It is preferred that the immunization method be at least 20% effective in preventing death in an immunized population after challenge with hantavirus. More preferably, the vaccination method is 50% or more effective, and most preferably 70-100% effective, in preventing death in an immunized population. The vaccination method is shown herein to be 100% effective in the hamster model for hantavirus. Hamsters have been used extensively as the laboratory model of choice for assessment of protective immune responses to hantaviruses. In contrast, unimmunized animals are uniformly infected by challenge with hantavirus. Our results indicate that vaccination with SEOV M genome segment protects against Hantaan virus (HTNV), and against Dobrava virus (DBOV).

Generally, the DNA vaccine administered may be in an amount of about 1-5 ug of DNA per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine for eliciting an immune response against one or more viruses, may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

In another embodiment, the present invention provides reagents useful for carrying out the present process. Such reagents comprise a DNA fragment containing M or S or both gene segments from hantavirus, and a small, inert, dense particle. The DNA fragment, and dense particle are those described above.

Preferably, the DNA is frozen or lyophilized, and the small, inert, dense particle is in dry powder. If a coating solution is used, the dry ingredients for the coating solution may be premixed and premeasured

and contained in a container such as a vial or sealed envelope.

The present invention also provides kits which are useful for carrying out the present invention.

- 5 The present kits comprise a first container means containing the above-described frozen or lyophilized DNA. The kit also comprises a second container means which contains the coating solution or the premixed, premeasured dry components of the coating solution.
- 10 The kit also comprises a third container means which contains the small, inert, dense particles in dry powder form or suspended in 100% ethanol. These container means can be made of glass, plastic or foil and can be a vial, bottle, pouch, tube, bag, etc. The
- 15 kit may also contain written information, such as procedures for carrying out the present invention or analytical information, such as the amount of reagent (e.g. moles or mass of DNA) contained in the first container means. The written information may be on
- 20 any of the first, second, and/or third container means, and/or a separate sheet included, along with the first, second, and third container means, in a fourth container means. The fourth container means may be, e.g. a box or a bag, and may contain the
- 25 first, second, and third container means.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

30

The following materials and method were used in the examples below.

MATERIALS AND METHODS

Viruses, cells, and medium

- 5 SEOV, strain SR-11 (Kitamura *et al.*, 1983, *Jpn. J. Med. Sci. Biol.* 36, 17-25) was propagated in Vero E6 cells (Vero C1008; ATCC CRL 1586). Transient expression experiments were performed with COS cells (COS-7; ATTC CRL1651). Both cell types were
- 10 maintained in Eagle's minimal essential medium with Earle's salts (EMEM) containing 10% fetal bovine serum, 10 mM HEPES pH 7.4; and antibiotics (penicillin [100 U/ml], streptomycin [100 µg/ml], and gentamycin [50 µg/ml]) (cEMEM).
- 15 **Construction of SEOV M and S naked DNA plasmids**
RT-PCR cloning of the M and S genome segments of SEOV, strain SR-11, was described previously (Arikawa *et al.*, 1990, *Virology* 176, 114-125). The cDNA representing each genome segment was subcloned into
- 20 the *Not* I or *Bam* HI site of a previously described naked DNA vector pWRG7077 (Schmaljohn *et al.*, 1997, *J. Virol.* 64, 3162-3170). Plasmid DNA was purified using Qiagen maxiprep DNA purification kits according to the manufacturer's directions (Cat. no. 12163, Qiagen).
- 25 The DNA we used for both intramuscular needle inoculation and epidermal gene gun inoculation was not prepared using endo-free qiagen DNA purification kits, and therefore, the DNA was not endotoxin-free (Qiagen plasmid kits yield ~9.3 endotoxin units per µg DNA
- 30 [QIAGEN Plasmid Purification Handbook 01.97]). To control for possible immunostimulatory effects of endotoxin, our negative control plasmid DNA was prepared in precisely the same was as the vaccine candidates.

Indirect fluorescent antibody test (IFAT)

- IFAT was a modification of a previously described procedure (Kamrud et al., 1995, *Exp. Parasitol.* 81, 394-403). COS cells grown on 15mm glass cover slips
- 5 in 12-well cell culture plates were transfected with 1 μ g of plasmid DNA using Fugene6 (Boehringer Mannheim) as described by the manufacturer. Twenty-four hr posttransfection, cover slips were rinsed once with PBS (pH 7.4), fixed with acetone for 10 min at room
- 10 temperature, and then reacted with anti-SEOV polyclonal antiserum (rabbit), or sera from vaccinated animals. Antibodies were diluted in PBS containing 3% fetal bovine serum (FBS) and then incubated on transfected cells for 30 min at 37°C. Cover slips were
- 15 rinsed three times with PBS and incubated for 30 min at 37°C with biotinylated donkey anti-rabbit followed by streptavidin conjugated fluorescein (Amersham), or fluorescein-labeled goat anti-hamster antibody (Kirkegaard & Perry Laboratories) diluted in PBS, 3%
- 20 FBS. Evans blue (Fisher) was included in the secondary antibody solution as a counter stain. Cover slips were rinsed three times with PBS and once with deionized water and then were placed on a drop of fluorescence mounting medium (DAKO) on glass slides.
- 25 Cells were observed with a Zeiss Axioplan fluorescence microscope.

Immunoprecipitation

- COS cells grown in T-25 cell culture flasks were transfected with 8 μ g of plasmid DNA using Fugene6
- 30 (Boehringer Mannheim). After 24 hr, expression products were radiolabeled with Promix ($[^{35}\text{S}]$ -methionine and $[^{35}\text{S}]$ -cysteine, Amersham) and immunoprecipitated as described previously (Schmaljohn et al., 1997, *supra*).

Vaccination with the gene gun

Cartridges for the gene gun were prepared as described previously (Eisenbraun *et al.*, 1993, *DNA Cell. Biol.* 12, 791-797; Schmaljohn *et al.* 1997, supra). Briefly, ~1.5 µg of DNA was precipitated onto 0.5 mg of ~ 2 µm diameter gold beads (Degussa). DNA-coated gold beads were used to coat the inner surface of Tefzel tubing (McMaster-Carr). Cartridges were made by cutting the DNA-gold-coated Tefzel tubing into ~0.5-inch sections, each containing ~0.5 mg of gold, coated with ~0.75 µg of DNA (~50% of the precipitated DNA bound to the gold as determined by elution and fluorometric assay). To vaccinate animals, abdominal fur was removed with clippers and DNA-coated gold was administered to nonoverlapping sites on the abdominal epidermis by using the gene gun (Powderject-XR delivery device, Powderject Vaccines, Inc.) as described previously (Pertmer *et al.*, 1995, *Vaccine* 13, 1427-1430). BALB/c mice (National Cancer Institute) and outbred Syrian hamsters (Charles River) were vaccinated with two or four cartridges, respectively, under 400 p.s.i. of helium pressure. This research was conducted in accordance with procedures described in the Guide for the Care and Use of Laboratory Animals (National Institute of Health, 1996). The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Vaccination by intramuscular needle inoculation

Plasmid DNA (25 µg) suspended in 50 µl of PBS (pH 7.4) was injected into the gastrocnemius muscle of anesthetized mice with a 28.5-gauge needle.

ELISA

To detect SEOV G1 and G2, ELISA plates (Costar) were coated with SEOV-infected Vero E6 cell lysates

[¹²⁵I]-irradiated (3 million rads) to inactivate virus] diluted in carbonate buffer (pH. 9.6) overnight at 4°C. Plates were blocked with PBS, 3% skim milk, 0.05% Tween-20 (blocking solution) for 1 hr at 37°C

5 and then washed once with PBS, 0.05% Tween-20 (wash solution). Mouse or hamster sera diluted in blocking solution were added to the wells, and plates were incubated as before. Plates were washed four times with wash solution and then incubated for 1 hr with

10 horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cat. no. A-4416, Sigma) at a dilution of 1/1,000 or peroxidase-labeled goat anti-hamster antibody (Cat. no. 14-22-06, Kirkegaard & Perry Laboratories) diluted 1/2,000 in blocking solution.

15 Plates were washed as before and then incubated for 10 min at room temperature with tetra-methylbenzidine substrate (TMB, Cat. no. 50-76-04, Kirkegaard & Perry Laboratories). The colorimetric reaction was stopped by adding Stop solution (Cat. no. 50-85-04, Kirkegaard

20 & Perry Laboratories) and the optical density (O.D.), 450 nm, was determined. The method for detecting SEOV N-specific antibodies was adapted from a previously described technique (Elgh *et al.*, 1997, *J. Clin. Microbiol.* 35, 1122-1130). Amino acids 1-117 of the

25 nucleocapsid protein of SEOV (strain SR-11) were expressed as a histidine-tagged fusion protein by using the pRSET plasmid (Invitrogen) in *Escherichia coli* BL21 (DE3) (Novagen, Inc.) and purified by affinity chromatography on Ni-NTA columns (Qiagen).

30 Antigen was diluted in carbonate buffer (pH 9.6), added to 96-well microtiter plates (Maxisorp; NUNC) (0.2 µg in 100 µl per well), and incubated overnight at 4°C. Plates were washed in deionized water and incubated with blocking solution for 30 min at room

35 temperature. After plates were washed in deionized

water, 100 μ l of serum (diluted in blocking buffer containing *E. coli* antigen extract [0.6 mg/ml]) was added to duplicate wells. Plates were incubated at 37°C for 1 h and again washed as before. Addition of HRP secondary antibody and colorimetric detection were added as described above for the SEOV G1-G2 ELISA. End-point titers were determined as the highest dilution with an O.D. greater than the mean O.D. value of serum samples from negative control plasmid (pWRG7077)-vaccinated animals plus three standard deviations.

Plaque reduction neutralization tests (PRNT)

Neutralization assays were performed essentially as previously described (Chu et al., 1995, *J. Virol.* 69, 6417-6423). Serum samples were diluted in cEMEM and then combined with an equal volume (111 μ l) of cEMEM containing ~75 PFU of SEOV. This serum-virus mixture was incubated overnight at 4°C and then 200 μ l/well was added to 6well plates containing Vero E6 monolayers 3-7 days old. After a 1-hr adsorption at 37°C, 3 ml of overlay medium (Earl's basal minimal essential medium (EBME), 10 mM HEPES, 0.6% agarose [Sea Kem ME agarose], 8 mM L-glutamine, antibiotics) containing 10% FBS and 1x nonessential amino acid mixture (GIBCO BRL) was added to each well. Plates were incubated at 37°C for 7 days and then stained by adding 2 ml/well of overlay medium containing 5% FBS and 5% neutral red solution (GIBCO BRL). Plaques were counted after 2 days at 37°C. Serum samples were heat inactivated (56°C, 30 min) before assay. In experiments where complement was used, serum and virus were incubated overnight in the presence of 5% guinea pig complement (Cat. no. ACL-4051, Accurate Chemical and Scientific Corporation).

Challenge with SEOV

Syrian hamsters were injected intramuscularly (caudal thigh) with a 25-gauge needle with 10^3 PFU of SEOV diluted in 0.2 ml sterile PBS (pH 7.4). The 10^3 PFU dose was based on previously published work that reported 100% infection of hamsters after this challenge dose and route (Schmaljohn *et al.*, 1990, *supra*; Chu *et al.*, 1995, *supra*). Twenty-eight days after challenge, the hamsters were anesthetized and exsanguinated by cardiac puncture. Postchallenge serum was evaluated for the presence of anti-SEOV antibody by IFAT, ELISA, and PRNT. A dramatic rise in postchallenge antibody titer to viral proteins other than the immunogens indicated that the hamster was infected with SEOV.

Cross protection assay. Groups of 4-5 hamsters were vaccinated with either pWRG/SEO M or negative control plasmid (pWRG7077). Vaccinations consisted of four gene gun administrations (~1.5 ug DNA per administration) three times at three week intervals. Three weeks after the final vaccination, prechallenge serum samples were obtained and the hamsters were challenged with 1,000 PFU of Hantaan virus, 1,000 PFU of Dobrava virus, or 1,000-2,000 PFU of Puumala virus. Twenty-eight days after challenge postchallenge serum samples were obtained. Pre and postchallenge serum samples were evaluated by anti-N ELISA to detect antibody to nucleocapsid, by a Hantaan, Dobrava, or Puumala plaque reduction neutralization test (PRNT) to detect the resective neutralizing antibodies depending on the challenge virus. Details of vaccination procedures, anti-N ELISA, and PRNT are as described in Hooper *et al*, Virology, March 15,1999.

EXAMPLE 1

Transient expression of hantavirus genes.

The cDNA representing the M (SEQ ID NO:1) or S (SEQ ID NO:2) gene segment of the SR-11 strain of SEOV was subcloned into the naked DNA expression vector pWRG7077 downstream of the cytomegalovirus immediate early promoter to yield pWRG/SEO-M (SEQ ID NO:3) and pWRG/SEO-S (SEQ ID NO:4), respectively (Fig. 1). To assess expression by the M and S constructs, these plasmids were transfected into COS cells and the expressed proteins were either immunostained or immunoprecipitated. The presence of stained cells in transfected monolayers incubated with anti-SEOV polyclonal sera indicated that SEOV reactive protein(s) were expressed from both pWRG/SEO-M and pWRG/SEO-S (Fig. 2A). Radiolabeled expression products were immunoprecipitated with anti-SEOV polyclonal antisera and analyzed by SDS-PAGE (Fig. 2B). Immunoprecipitated proteins had apparent molecular sizes expected for G1, G2, and N (80 kDa, 56 kDa, and 48 kDa) (Elliot et al. 1984, *J. Gen. Virol.* 65, 1285-1293; Schmaljohn et al. 1986, *Virology* 155, 633-643). Additional protein bands with apparent molecular masses of ~37 kDa and ~28 kDa were co-precipitated with N. These polypeptides were immunoprecipitated by a N amino-terminus-specific monoclonal antibody, and could be detected by Western blot, suggesting they represent truncated forms of N (data not shown).

EXAMPLE 2

Vaccination of mice with pWRG/SEO-M or pWRG/SEO-S. To determine if either the M or S construct could elicit an antibody response in mice, groups of 10 6- to 8-week-old female BALB/c mice were vaccinated with pWRG/SEO-M, pWRG/SEO-S, or negative

control DNA (pWRG7077) by two different routes: inoculation of the epidermis by gene gun, or inoculation of the gastrocnemius muscle by needle injection. Mice received a priming vaccination followed by two boosts at 4 week intervals. Blood samples collected before each vaccination and 3 weeks after the final boost were screened for a SEOV-specific antibody response by ELISA. Both the M and S constructs elicited a SEOV-specific antibody response by both routes of DNA administration (Fig. 3). Most mice vaccinated with SEOV S by gene gun exhibited specific antibodies after only one vaccination, and the antibody responses increased after subsequent vaccinations (Fig. 3B). To compare the relative antibody responses, end-point ELISA titers were determined for the final blood samples (Table 1). Gene gun vaccination resulted in higher seroconversion frequencies and geometric mean titers (GMT) than intramuscular vaccination with either construct.

Table 1: ELISA Titers in Mice Vaccinated with SEOV Naked DNA Vaccines

	Inoculated DNA	Vaccine route ^a	Positive/ total	End point titer range	GMT ^b
	pWRG/SEO-M	g.g.	9/10	200-1600	303
25	pWRG/SEO-M	i.m.	7/10	100-400	132
	pWRG/SEO-S	g.g.	10/10	6400-12800	7352
	pWRG/SEO-S	i.m.	9/10	800-6400	1213

Note. Mice were vaccinated three times at 4-week intervals. Titers were determined for sera collected three weeks after the final boost.

^ag.g. indicates gene gun; i.m., intramuscular needle inoculation.

^bGeometric mean titer of all animals in group.

To determine if naked DNA vaccination elicited neutralizing antibodies, we performed plaque reduction neutralization tests (PRNT) on sera collected 3 weeks after the final boost. All (10/10) mice that were vaccinated with pWRG/SEO-M by gene gun exhibited PRNT_{50%} titers ranging from 40 to 1280 (Fig. 4). Only 1/10 mice vaccinated with pWRG/SEO-M by needle injection exhibited a detectable neutralizing antibody response (PRNT_{50%} = 40). Mice vaccinated with pWRG/SEO-S or pWRG7077 by either gene gun or needle inoculation did not develop neutralizing antibodies (Fig. 4).

EXAMPLE 3

Vaccination and challenge of hamsters. To study the protective efficacy of the naked DNA hantavirus vaccines, groups of five hamsters were vaccinated with either pWRG/SEO-M, pWRG/SEO-S, or negative control plasmid pWRG7077 by gene gun (a priming vaccination followed by two boosts at 4-week intervals) and then challenged with SEOV 6 weeks after the final boost. IFAT, ELISA, and PRNT were performed on serum samples taken before vaccination, on the day of challenge, and 4 weeks after challenge. Because there are no disease models for hantavirus in adult rodents, we evaluated whether or not our DNA vaccines could protect against infection rather than prevent disease.

IFAT and ELISA results demonstrated that before vaccination, none of the hamsters was positive for either SEOV G1-G2 or N protein (data not shown). After vaccination (prechallenge sera drawn on day of challenge), five of five hamsters vaccinated with pWRG/SEO-M developed anti-G1-G2 antibodies, and four of five hamsters vaccinated with the pWRG/SEO-S exhibited anti-N antibodies by IFAT (Table 2). The

prechallenge ELISA results detected similar antibody responses except that only four of five pWRG/SEO-M vaccinated hamsters were positive in the anti-SEOV ELISA. The pWRG7077 vaccinated hamsters failed to develop both G1-G2- and N-specific antibodies by IFAT and ELISA before challenge.

After challenge, serum samples from hamsters previously vaccinated with pWRG/SEO-M remained negative for anti-nucleocapsid antibody by IFAT indicating the animals were not infected with SEOV. In contrast, postchallenge serum samples from pWRG/SEO-S and pWRG7077 vaccinated hamsters were positive for both G1-G2 and N antibodies, indicating that the animals were infected when challenged with SEOV. Postchallenge ELISA results were similar to the IFAT results; however, three of five of the pWRG/SEO-M vaccinated hamsters exhibited a weak anti-N response after challenge indicating that, although the IFAT were negative, the hamsters had developed antibodies to the challenge virus.

Before vaccination, none of the hamsters had SEOV neutralizing antibody titers (data not shown). After vaccination, all hamsters vaccinated with pWRG/SEO-M developed neutralizing antibodies with PRNT_{80%} titers ranging from 160 to 640 (Table 2). Neutralizing antibody was not detected in pWRG/SEO-S and pWRG7077 prechallenge sera from vaccinated hamsters. After SEOV challenge, neutralizing antibody titers of the pWRG/SEO-M immunized animals remained essentially the same as before challenge (plus or minus twofold). In contrast, the pWRG7077 and pWRG/SEO-S postchallenge neutralizing antibody titers increased significantly from <20 to between 1,280 and >81,920.

Table 2. Serological responses and protection of hamsters vaccinated with naked DNA vaccines.

	Vaccine _a	Hamster	IFAT _b		anti-N		anti-G1-G2		PRNT _{50%}		Protection
			pre		post		pre		post		
			pre	post	pre	post	pre	post	pre	post	
5	pWRG/SEO-M	1	-	-	+	+	+	+	320 (20)	640	yes
		2	-	-	+	+	+	+	320 (40)	640	yes
		3	-	-	+	+	+	+	320 (20)	1280	yes
		4	-	-	+	+	+	+	80 (<20)	160	yes
		5	-	-	+	+	+	+	640 (20)	1280	yes
10	PWRG/SEO-S	1	+	+	-	+	+	+	<20 (<20)	>640	no
		2	+	+	-	+	+	+	<20 (<20)	>640	no
		3	+	+	-	+	+	+	<20 (<20)	>640	no
		4	+	+	-	+	+	+	<20 (<20)	>640	no
		5	-	+	-	+	+	+	<20 (<20)	>640	no
15	PWRG7077	1	-	+	-	+	+	+	<20 (<20)	>640	no
		2	-	+	-	+	+	+	<20 (<20)	>640	no
		3	-	+	-	+	+	+	<20 (<20)	>640	no
		4	-	+	-	+	+	+	<20 (<20)	>640	no
		5	-	+	-	+	+	+	<20 (<20)	>640	no
20	PWRG7077	1	-	+	-	+	+	+	<20 (<20)	>640	no
		2	-	+	-	+	+	+	<20 (<20)	>640	no
		3	-	+	-	+	+	+	<20 (<20)	>640	no
		4	-	+	-	+	+	+	<20 (<20)	>640	no
		5	-	+	-	+	+	+	<20 (<20)	>640	no

^a Three vaccinations consisting of 4 gene gun cartridges (about 1.5 ug/cartridge) administered at 4 week intervals.

^b Sera were screened at 1:200 in IFAT using PWRG/SEO-S or PWRG/SEO-M transfect cells as target antigen for anti-n and anti-G1-G2, respectively. - indicates no reactivity; + indicates reactivity.

^c PRNT values are reciprocal serum dilutions reducing plaque number by 50%. Assays were performed in the presence of 5% guinea pig complement. Numbers in parenthesis are PRNT values of heated serum in the absence of complement.

pre, prechallenge serum samples from day of challenge; post, postchallenge serum samples from 4 weeks post challenge.

During the course of this study, we observed that PRNT titers decreased dramatically if sera were heat-inactivated (56°C, 30 min). To test the possibility that the neutralizing activity was complement-enhanced, we performed PRNT assays in the presence of 5% guinea pig complement and found that adding complement increased the PRNT titers of heated serum 4- to 32-fold (Table 2). Complement alone had no effect on SEOV plaque number (data not shown).

Example 4

Vaccination with pWRG/SEO-M cross-protects against infection with Hantaan virus

Hamsters vaccinated with pWRG/SEO-M had no detectable anti-N antibody prior to challenge, and the postchallenge anti-N response was undetectable or very low ($\leq 1:200$) (Fig. 6). In contrast, hamsters vaccinated with the negative control plasmid exhibited a dramatic rise in anti-N antibody (~ 2 log increase over background). The PRNT indicated that vaccination with pWRG/SEO-M elicited detectable levels of cross neutralizing antibodies in 3 of 4 hamsters. The PRNT titers of these hamsters did not rise after challenge. In the one hamster with undetectable ($< 1:20$) Hantaan neutralizing antibody, the PRNT titer did rise; however, the increase was at least 32-fold lower than that seen in the hamsters vaccinated with the negative control plasmid.

The low or absent increase in antibody titers to Hantaan structural proteins (as measured by ELISA and PRNT) after challenge with Hantaan virus indicates that vaccination with the Seoul virus M gene (pWRG/SEO-M) confers protection against infection with Hantaan virus.

Example 5**Vaccination with pWRG/SEO-M cross-protects against infection with Dobrava virus.**

Hamsters vaccinated with pWRG/SEO-M had no detectable anti-N antibody prior to challenge, and the postchallenge anti-N response was undetectable. In contrast, all but one of the hamsters vaccinated with the negative control plasmid exhibited a dramatic rise in anti-N antibody (postchallenge anti-N titers of between 1:200 and 1:204,800). The PRNT indicated that vaccination with pWRG/SEO-M elicited low levels of cross neutralizing antibodies in 5 of 5 hamsters. The PRNT titers of these hamsters did not rise above 1:80 after challenge. In contrast, all but one hamster vaccinated with the negative control plasmid exhibited a high postchallenge PRNT (1:5120) indicating the hamsters were infected with Dobrava virus. It remains unclear why animal #182 was not infected.

The low or absent increase in antibody titers to Dobrava structural proteins (as measured by ELISA and PRNT) after challenge with Dobrava virus indicates that vaccination with the Seoul virus M gene (pWRG/SEO-M) confers protection against infection with Dobrava virus.

Example 5**Vaccination with pWRG/SEO-M fails to cross-protect against infection with Puumala virus.**

Hamsters vaccinated with pWRG/SEO-M had no detectable anti-N antibody prior to challenge. After challenge, all but one hamster had postchallenge anti-N response greater than or equal to 1:200 indicating the hamsters were infected with Puumala virus. The negative control hamsters had similar pre and postchallenge anti-N titers. The PRNT results

indicated that, although vaccination with pWRG/SEO-M elicited neutralizing antibodies against Seoul virus (empty circles), these antibodies did not cross-neutralizing Puumala virus, and failed to protect
5 against infection. The postchallenge Puumala virus PRNT titers of the pWRG/SEO-M vaccinated and negative control vaccinated hamsters were all similar (greater than or equal to 1:160), except one negative control hamster that was not infected. It remains unclear why
10 negative control animal #220 was not infected.

The increase in antibody titers to Puumala virus structural proteins (as measured by ELISA and PRNT) after challenge with Puumala virus indicates that vaccination with the Seoul virus M gene (pWRG/SEO-M)
15 fails to confers protection against infection with Puumala virus. It should be noted that the hamster with the highest prechallenge anti-Seoul virus PRNT titer was 1:640 (animal #209). It is possible that animals with higher prechallenge neutralizing antibody
20 titers might be cross-protected against Puumala virus.

DISCUSSION

We report that naked DNA shows promise as a way to vaccinate against hantaviruses. Our results demonstrate that vaccination with cDNA representing
25 either the SEOV M or S genome segment elicited antibody responses in BALB/c mice and Syrian hamsters. Hamsters vaccinated with DNA expressing the M segment, but not S segment, developed neutralizing antibodies and were protected against infection with SEOV.

30 In our initial immunogenicity experiments, gene gun vaccination of BALB/c mice resulted in higher seroconversion rates than intramuscular needle inoculation for both pWRG/SEO-M and pWRG/SEO-S, as measured by ELISA and PRNT. These preliminary data
35 prompted us to focus on the gene gun rather than the

intramuscular route of DNA delivery. However, because only one intramuscular DNA dose was tested (25 μ g per animal), it is possible that the seroconversion rate and/or titers could be increased by adjusting

5 parameters such as the amount of DNA injected, site of injection, or vaccination schedule. It is also possible that needle inoculation elicited a predominantly cell-mediated rather than humoral response to the DNA vaccination, as has been seen by

10 others (Feltquate et al., 1997, *J. Immunol.* 158, 2278-2284; Robinson and Torres, 1997, *Semin. Immunol.* 9, 271-283; Gregoriadis, 1998, *Pharm. Res.* 15, 661-670).

Gene gun vaccination with pWRG/SEO-M protected all hamsters against infection with SEOV as measured

15 by 1) undetectable, or barely detectable, postchallenge anti-N antibody response and, 2) PRNT titers that remained essentially unchanged after SEOV challenge. Although the IFAT results indicated that none of the pWRG/SEO-M vaccinated hamsters developed

20 anti-N antibodies after challenge, the more sensitive anti-N ELISA revealed that three hamsters had a weak anti-N antibody response. Because the postchallenge anti-N ELISA GMT of these hamsters (GMT = 50) was more than 50 times lower than the negative control pWRG7077

25 vaccinated hamsters (GMT > 3,195), it is likely the anti-N antibodies represent an antibody response to either the input SEOV antigen (no replication), or to a limited infection (e.g., one round of replication). The PRNT data provided additional evidence that

30 vaccination with pWRG/SEO-M, but not pWRG/SEO-S, protected hamsters from SEOV infection. Whereas the negative control pWRG7077-vaccinated hamsters' PRNT titers increased from <20 to between 10,280 and 81,920 after challenge, the pWRG/SEO-M-vaccinated hamster

35 PRNT titers remained within twofold of prechallenge

levels. Moreover, the postchallenge PRNT titers of the pWRG/SEO-M-vaccinated hamsters (GMT = 368) remained well below the postchallenge PRNT titers of the pWRG7077-vaccinated hamsters (GMT > 27,024).

- 5 Hamsters vaccinated with pWRG/SEO-S were infected with SEOV after challenge. The pWRG/SEO-S-vaccinated hamsters' postchallenge PRNT titers increased from <20 to between 2,560 and 20,480. Although the postchallenge PRNT titers of the pWRG/SEO-S were high
10 (GMT = 5,885), it is noteworthy that these titers were approximately fivefold lower than the negative control (pWRG7077) postchallenge titers (GMT > 27,024).

- The absence of a neutralizing antibody response in mice and hamsters vaccinated with pWRG/SEO-S, with
15 or without complement, is consistent with published data that monoclonal antibodies to G1 and G2, but not N, have neutralizing activity (Dantas et al., 1986, *Virology* 151, 379-384; Arikawa et al., 1989, *J. Gen. Virol.* 70, 615-624; Schmaljohn et al., 1990, *J. Virol.* 64, 3162-3170; Arikawa et al., 1992, *Arch. Virol.* 70, 615-624), and that vaccination with vaccinia
20 recombinants expressing G1 and/or G2, but not N, elicit a neutralizing response (Pensiero et al., 1988, *J. Virol.* 62, 696-702; Schmaljohn et al. 1992, *Vaccine* 10, 10-13; Xu et al., 1992, *Am. J. Trop. Med. Hyg.* 47, 397-404; Chu et al., 1995, supra).

- Failure of an anti-N immune response to protect against infection was unexpected because others found that vaccination with recombinant N expressed in
30 insect cells, bacteria, or as chimeric HEV core particles, protect against infection (Schmaljohn et al., 1990, supra; Yoshimatsu et al., 1993, *Arch. Virol.* 130, 365-376; Lundkvist et al., 1996, *Virology* 216, 397-406; Ulrich et al., 1998, *Vaccine* 16, 272-
35 280). Also, vaccination with recombinant vaccinia

viruses expressing the SEOV S segment partially protected gerbils from SEOV infection (Xu et al. 1992, supra). Failure of naked DNA vaccination with the S segment to protect hamsters in our experiments may be related to a quantitative rather than qualitative deficiency. Although four of five hamsters vaccinated with pWRG/SEO-S developed a detectable antibody response by IFAT and ELISA, the titers were relatively low (ELISA titers less than or equal to 1:1,600). The reason for these low anti-N antibody titers in hamsters, but not mice, remains unclear. Fault cannot lie with the gene gun cartridges as the same lot was used to vaccinate both hamsters and mice on the same day; and the mouse seroconversion rate was 90%, with an ELISA GMT of over 7,000. Low antibody titers may reflect a poor overall immune response to vaccination with pWRG/SEO-S in hamsters or, alternatively, it is possible that epidermal administration of S DNA with the gene gun results in a predominantly cell-mediated, rather than humoral, response to N. Nevertheless, our findings demonstrate that there was a detectable anti-N immune response in four of five hamsters after naked DNA gene gun vaccination with pWRG/SEO-S, and that this response, whether humoral and/or cell-mediated, was insufficient to protect against SEOV infection. The ~fivefold difference in postchallenge PRNT titers of pWRG/SEO-S versus pWRG7077 vaccinated hamsters suggests pWRG/SEO-S vaccination may nonetheless limit SEOV infection.

We found that SEOV neutralizing activity elicited in mice and hamsters was reduced 4- to 32-fold if the sera was heat inactivated (56°C, 30 min). This loss in neutralizing activity could be completely reversed if guinea pig complement was included in the PRNT.

These data are consistent with those of others who

reported that hantavirus neutralizing activity in sera from humans, mice, and especially rats, was enhanced by complement (Takenaka *et al.*, 1985, *Arch. Virol.* 84, 197-206; Asada *et al.*, 1989, *J. Gen. Virol.* 70, 819-825). The observation that complement plays a major role in hantavirus neutralization is notable because PRNT titers are commonly used to assess the immunogenicity and predicted efficacy of hantavirus vaccines. Conditions that deplete or add complement, such as heat inactivation or addition of exogenous complement (e.g., as a purified protein fraction or as fresh animal sera), could alter PRNT titers and, therefore, could alter the assessment of candidate vaccines.

Future studies are aimed at developing naked DNA vaccines that protect against other HFRS-associated hantaviruses, including HTNV, DOBV, and PUUV; and also against the recently discovered North American hantaviruses associated with hantavirus pulmonary syndrome (reviewed in Schmaljohn and Hjelle, 1997, *Emerg. Infect. Dis.* 3, 95-104). We are also interested in evaluating the protective efficacy of candidate vaccines against increasing challenge doses, as well as different routes of challenge.

25

30

What is claimed is:

1. A composition of matter comprising a carrier particle; and a DNA sequence coated onto the carrier particle, the DNA sequence comprising a promoter
5 operative in the cells of a mammal and a protein coding region coding for a determinant of a hantavirus protein.
2. The composition of claim 1 wherein said protein coding region encodes a protein selected from
10 the group consisting of M gene segment proteins and S gene segment proteins.
3. The composition of claim 1 wherein said hantavirus is chosen from the group consisting of
15 Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus..
4. The composition of claim 3 wherein said hantavirus is Seoul virus.
- 20 5. The composition of claim 4 wherein the protein coding region comprises SEQ ID NO:1.
6. The composition of claim 4 wherein the protein coding region comprises SEQ ID NO:2.
7. The composition of claim 1, wherein said DNA
25 sequence comprises pWRG-SEO-M.
8. The composition of claim 1, wherein said DNA sequence comprises pWRG-SEO-S.
9. A method for inducing a protective immune
30 response to a hantavirus protein in a mammal, comprising
 - (i) preparing a nucleic acid encoding a determinant of a hantavirus protein operatively linked to a promoter operative in cells of a mammal;
 - (ii) coating the nucleic acid in (i) onto carrier
35 particles;

(iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and

(iv) detecting a protective immune response in said mammal upon exposure to a hantavirus.

5 10. The method according to claim 9 wherein the carrier particles are gold.

11. The method according to claim 9 wherein the protein determinant is chosen from the group consisting of M genome segment proteins and S segment
10 proteins.

12. The method according to claim 9 wherein said hantavirus is chosen from the group consisting of Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus,
15 Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

13. The method of claim 12 wherein said hantavirus is Seoul virus.

14. The method according to claim 13 wherein said
20 nucleic acid comprises SEQ ID NO:1.

15. The method according to claim 13 wherein said nucleic acid comprises SEQ ID NO:2.

16. The method according to claim 13 wherein said nucleic acid comprises SEQ ID NO: 1 and SEQ ID NO:2.
25

17. A method for inducing a protective immune response to a hantavirus infection in a mammal comprising

(i) preparing a nucleic acid encoding a determinant of a first hantavirus protein operatively
30 linked to a promoter operative in cells of a mammal;

(ii) coating the nucleic acid in (i) onto carrier particles;

(iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and

(iv) detecting an immune response in said mammal upon a exposure to a second hantavirus.

18. The method according to claim 17 wherein said first hantavirus is SEOV.

5 19. The method according to claim 18 wherein said second hantavirus is Dobrava virus.

20. The method according to claim 18 wherein said second hantavirus is Hantaan virus.

21. The method according to claim 16 wherein said
10 nucleic acid is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

22. A vaccine against hantavirus infection comprising the composition of claim 3.

23. A vaccine against hantavirus infection
15 comprising the composition of claim 4.

24. A vaccine against hantavirus infection comprising the composition of claim 5.

25. A vaccine against hantavirus infection comprising the composition of claim 6.

20 26. A multivalent vaccine for protection against infection with more than one hantavirus comprising a composition of matter comprising a carrier particle having one or more DNA sequence coated onto the carrier particle, the DNA sequence comprising a
25 promoter operative in the cells of a mammal and a protein coding region coding for a determinant of a first hantavirus protein said hantavirus selected from the group consisting of SEOV, Dobrava, Puumala, Hantaan, Sin Nombre virus, Black Creek Canal virus,
30 Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

27. The multivalent vaccine of claim 26, further comprising a composition comprising a carrier particle having one or more DNA sequence coated onto the
35 carrier particle, the DNA sequence comprising a

promoter operative in the cells of a mammal and a protein coding region coding for a determinant of a second hantavirus different from said first hantavirus, said second hantavirus selected from the group consisting of Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

10

15

20

25

30

ABSTRACT

Seoul virus (SEOV) is one of four known hantaviruses causing hemorrhagic fever with renal syndrome (HFRS). Candidate naked DNA vaccines for HFRS were constructed by subcloning cDNA representing the medium (M) (encoding the G1 and G2 glycoproteins) or small (S) (encoding the nucleocapsid protein) genome segment of SEOV into the DNA expression vector pWRG7077. We vaccinated BALB/c mice with three doses of the M or S DNA vaccine at 4-week intervals by either gene gun inoculation of the epidermis, or needle inoculation into the gastrocnemius muscle. Both routes of vaccination resulted in antibody responses as measured by ELISA; however, gene gun inoculation elicited a higher frequency of seroconversion, and higher levels of antibodies in individual mice. We vaccinated Syrian hamsters with the M or S construct using the gene gun and found hantavirus-specific antibodies in 5/5 and 4/5 hamsters, respectively. Animals vaccinated with the M construct developed a neutralizing antibody response which was greatly enhanced in the presence of guinea pig complement. Immunized hamsters were challenged with SEOV and, after 28 days, were monitored for evidence of infection. Hamsters vaccinated with M were protected from infection, but hamsters vaccinated with S were not protected.

Fig. 1

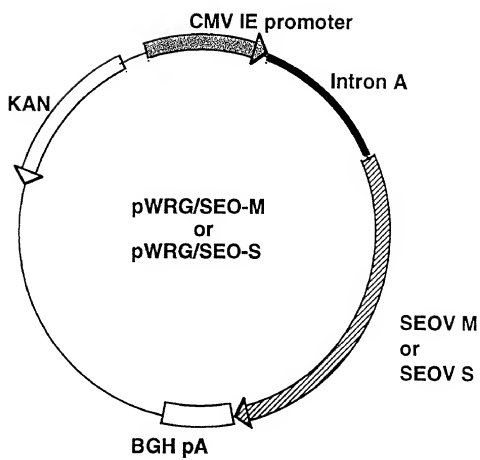
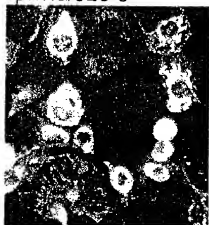


Fig. 2

A.

pWRG/SEO-S



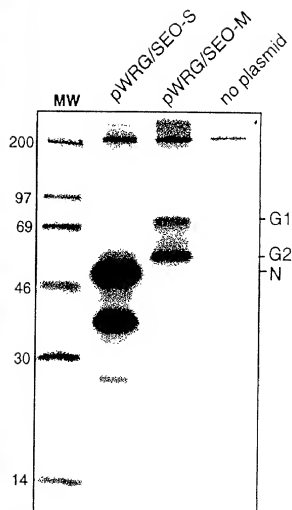
pWRG/SEO-M



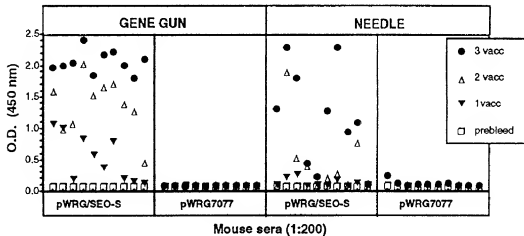
pWRG7077



B.



B. SEO-S



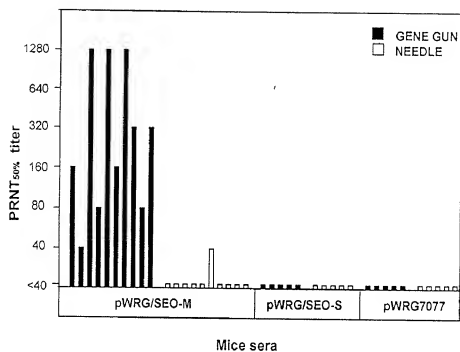


FIGURE 4

CROSS-PROTECTION AGAINST HANTAAIN

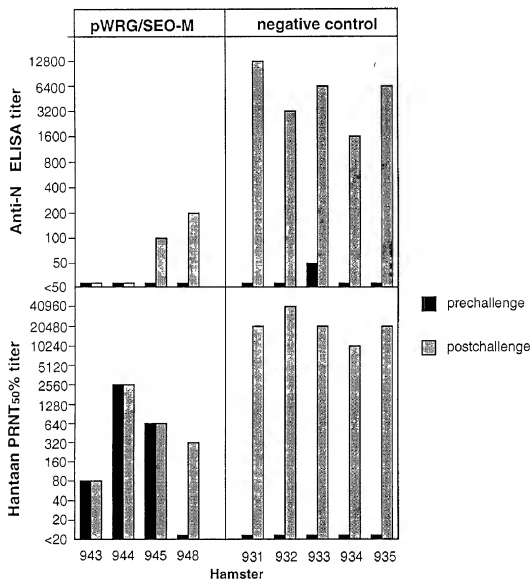


FIGURE 5

Vaccination with pWRG/SEO-M protects against Dobrava virus

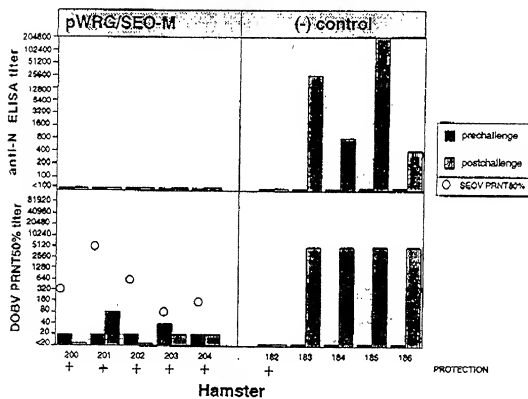


FIGURE 6

Vaccination with pWRG/SEO-M fails to protect against Puumala virus

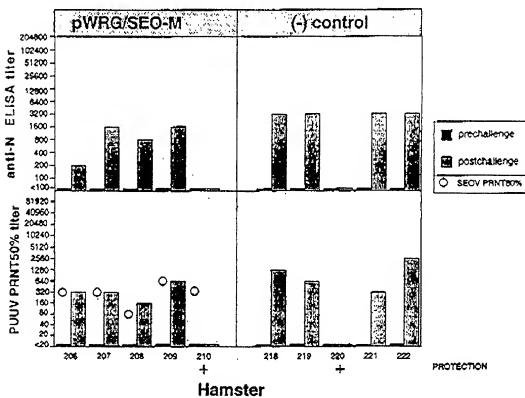


FIGURE 7

Schmaljohn and Hooper
Title: DNA Vaccines Against Hantavirus Infections

SEQ ID NO: 3 : pWRG/SEO-M

GGGGGGGGGGCGCTGAGGTCTGCCTCGTGAAGAAGGTGTGCTGACTCATACCAGGCCT
GAATCGCCCCATCATCCAG
CCAGAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTGTAGGTGGACCAGTTGGTGATT
TTGAACCTTTTGCTTTTGCCA
CGGAACGGTCTGCGTTTGTTCGGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTCG
ATTTATTCAACAAAGCCGC
CGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGTTACAACCAATTAACCAATTCGTGATTA
GAAAAACTCATCGAGCATC
AAATGAAACTGCAATTTATTTCATATCAGGATTATCAATACCATAATTTTGAAGCCGTT
TCTGTAATGAAGGAGAAAA
CTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGT
CCAACATCAATACCACTA
TTAATTTCCCTCGTCAAAAAAAGGTATCAAGTGAGAAATCACCATGAGTGACGACTGA
ATCCGGTGAGAATGGCAA
AGCTTATGCAATTTCTTCCAGACTTGTTCACAGGCCAGCCATTACGCTCGTCATCAAAAT
CACTCGCATCAACCAAAAC
GTTATTTCATTCGTGATTTCGCCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAA
TTACAAACAGGAATCGAAT
GCACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACTGAAATCAGGATATTC
TTCTAATACTCTGGAATGCT
GTTTTCCTGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCT
TGATGGTTCGGAAGAGGCAT
AAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCT
TTGCCATGTTTCAGAAAA
ACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTTCGCACTGATTGCCCACATT
ATCGCGAGCCCATTTATAC
CCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCTCGAGCAAGACGTTTCCCGTT
GAATATGGCTCATTAACACC
CCTTGATTACTGTTTATGTAAGCAGACAGTTTATTGTTTCATGATGATATATTTTATCT
TGTGCAATGTAACATCAGA
GATTTTGAGACACAACGTGGCTTTCCCCCCCCCCCCCGCATGCCTGCAGGTGCAGATAAAT
CAATATTTGGCTATTGGCCA
TTGCATACGTGTGATCTATATCATCAATATGTACATTTATATTGGCTCATGTCCAATATGAC
CGCCATGTTGACATTGATT
ATTGACTAGTTATTAATAGTAATCAATTACGGGGTCAATTAGTTCATAGCCCATATATGGAG
TTCCGCGTTACATAACTTA
CGGTAAATGGCCCGCTCGTGACCGCCCAACGACCCCGCCCATTGACGTCAATAATGACG
TATGTTCCCATAGTAACGC
CAATAGGACTTTCCATTGACGTCAATGGGTGGAGTATTACGGTAAACTGCCCACTTGGC
AGTACATCAAGTGTATCAT
ATGCCAAGTCCGGCCCTTATGACGTCAATGACGGTAAATGGCCCGCTTGGCATTTATGCC
CAGTACATGACCTTACGGG
ACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTT
TTGGCAGTACCAATGGG
CGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGA
GTTTGTTTTGGCACCAAAA
TCAACGGGACTTTCCAAAATGTCTGTAATAACCCCGCCCGTTGACGCAATGGGCGGTAGG
CGTGTACGGTGGGAGGTCT

ATATAAGCAGAGCTCGTTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTT
TGACCTCCATAGAAGACAC
CGGGACCGATCCAGGCTCCCGGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCA
AGAGTGACGTAAGTACCCG
CTATAGACTCTATAGGCACACCCCTTTGGCTCTTATGTCATGCTATACTGTTTTTGGCTTGG
GGCCTATACACCCCCGCTC
CTTATGCTATAGGTGATGGTATAGCTTAGCCTATAGGTGTGGGTATTATGACCATTATTGAC
CACTCCCCCTATTGGTGAGG
ATACTTTCCATTACTAACTCCATAACATGGCTCTTTGCCACAACATCTCTATTGGCTATAT
GCCAATACTCTGTCTCTCA
GAGACTGACACGGACTCTGTATTTTTACAGGATGGGGTCCATTTATTATTTACAAATTCA
CATATACAACACGCCGCTC
CCCCGTGCCCGCAGTTTTTATTAAACATAGCGTGGGATCTCCACGCGAATCTCGGGTACGT
GTTCCGGACATGGGCTCTT
CTCCGGTAGCGCGGAGCTTCCACATCCGAGCCCTGGTCCCATGCCTCCAGCGGCTCATGG
TCGCTCGGCAGCTCCTTGC
TCCTAACAGTGGAGGCCAGACTTAGGCACAGCACAAATGCCACCACCACCAAGTGTGCCGCA
CAAGGCCGTGGCGGTAGGG
TATGTGCTGAAAAATGAGCTCGGAGATTGGGCTCGCACCGTGACGCAGATGGAAGACTTAA
GGCAGCGGCAGAGAAGAT
GCAGGCAGCTGAGTTGTTGTATTCTGTATAAGAGTCAGAGGTAACCTCCCGTTCGCGGTGCTGT
TAA CGTGGAGGGCAGTGT
AGTCTGAGCAGTACTCGTTGCTGCCGCGCGCCACCAGACATAATAGCTGACAGACTAAC
AGACTGTTCCCTTTCCATGG
GTCCTTTCTGAGTCACCGTCCAAGCTTTCGCGCCGCGGATCTGCAGGAATTTCGGACAGAGA
GTAGTAGACTCCGCAAGAA
ACAGCAGTTAAAGAACATAGGATCATGTGGAGTTTGCTATTACTGGCCGCTTTAGTTGGC
CAAGGCTTTGCATTAAAAA
ATGTATTTGACATGAGAAATTCAGTTGCCCCACTCAGTCAACTTTGGGGAAAAAAGTGTGTC
AGGCTATACAGAATTTCCC
CCACTCTCATTACAGGAGGCAGAACAGCTAGTGCCAGAGAGCTCATGCAACATGGACAACC
ACCAGTCACTCTCAACAAT
AAATAAATTAAACCAAGGTCATATGGCGGAAAAAGCAAAATCAGGAATCAGCAAAACCAGAAT
TCATTTGAAGTTGTGAAA
GTGAAGTCAGCTTTAAAGGGTTGTGTATGTTAAAGCATAGAATGGTTGAAGAATCATATAG
AAATAGGAGATCAGTAATC
TGTTATGATCTAGCCTGTAATAGTACATTCTGTAAACCAACTGTTTATATGATTGTTCCCTA
TACATGCTTGCAACATGAT
GAAAAGCTGTTTGATTGGCCCTTGGCCCCACAGAAATCCAGGTTGTCTATGAAAGGACATAC
TGCACTACGGGTATATTGA
CAGAAGGAAAATGCTTTGTCCCTGACAAGGCTGTTGTCTAGTGCATTGAAAAGAGGCATGTA
TGCTATAGCAAGCATAGAG
ACAATCTGCTTTTTTATTCAACAGAAAGGGAATACATATAAGATAGTGACTGCCATTACAT
CAGCAATGGGCTCCAATG
TAATAATACAGATACTAAAGTTCAAGGATATTATATCTGTATTATTGGTGGAACTCCGCC
CCTGTATATGCCCTGCTG
GTGAAGACTTCAGAGCAATGGAGGTTTTTCTGGGATTATTACATCACCACATGGAGAAGA
CCATGACCTACCCGGCGAA
GAAATCGCAACGTACCAAGATTCAGGGCAGATAGAGGCAAAAATCCCTCATACAGTGAGCT
CCAAAAACTTAAATTTGAC
TGCTTTTGCAGGTATTTCCATCATCTCACTCACTAGTATATTGGCTGCTTCAGAAGATGGT
CGTTTTCATATTAGTCCCTG
GTTTATTTTCCCTAACCTAACATCAGTCAGTCTGTGACAACAAATGCACTCCCTTTAACTCTGGAG
GGGCCTAATTGATTTTAACG

GGATACTATGAGGCAGTCCACCCCTTGCAATGTGTTCTGTGCTCTTATCAGGACCAGGTGCTT
CATGTGAGGCCTTTTTCAGA
AGGAGGTATTTTCAATAATTACTTCTCCAATGTGTCGTGGTCTTAAGCAAAATAGATTTAGA
GCAGCTGAGCAGCAGATTA
GCTTTGTCTGCCAAAGAGTTGATATGGATATTATAGTGTACTGTAATGGTCAGAAAAAAC
AATCCTAACAAAAACATTA
GTTATAGGCCAATGTATTATATACTATTACAAGTCTCTTTTCACTGTTACCAGGGGTTGCC
ATTCTATTGCTATTGAGTT
GTGTGTTCCAGGGTTTTCATGGCTGGGCCACAGCTGCACCTTTTGATTACATTTCTGCTTCGGC
TGGGTATTGATTTCCTGCAT
GTACATTAGCTATTCTTTTAGTCTTAAAGTTCTTTGCAAAATATCCTTCATACAAGCAATCA
AGAGAACCGATTCAAAGCC
ATTCTACGGAATAAAGGAGAGTTTGAATAAACAAAGGGTTCCATGGTTTGTGAGATCT
GTAAGTATGAGTGTGAAAC
ATTAAAGGAATTGAAGGCACATAACCTATCATGTGTTCAAGGAGAGTGCCCATATTGCTTT
ACCCACTGTGAACCGACAG
AAACTGCAATTCAAGCACAATTACAAAGTTTGTCAAGCCACCCACCGATTGAGAGAAGATT
AAAAAAGACTGTAACTCTCT
CAAAATATTGGGCCAGGCTGTTACCGAACACTAAATCTTTTATAGGTATAAAAGTAGGTGTT
ATATTCTGACAAATGTGGAC
TCTTCTTCTCATATTGAATCCATCTCTGGCGACGAAGTGCAGCAGAAATCCCCCTTGTC
CCTCTCTGGACAGATAATG
CTCATGGCGTTGGGAGTGTCTCATGCATACGGATCTTGAATTAGACTTCTCTTTGCCATC
CAGTTCCTAAGTACACATAC
AAAAGACATCTCACAAAACCCAGTTAATGACCAACAGAGTGTCTCATTCATATAGAAATTG
AAAGTCAAGGCATTGGTGC
TGCTGTTTCATCATCTTTGGACATTGGTATGATGCAAGATTGAATCTAAAAACCTCATTTTCA
TGTTATGGTGCTGCAACAA
AATATCAATACCCATGGCACACTGCAAAATGCCATTTTGAAGAAATTATGAGTATGAA
TAGCTGGGCTTGCAACCCC
CCAGATTGCCCAGGGGTTGGTACAGGTTGTACTGCTTGTGGATTATATCTAGATCAATTGA
AGCCGGTAGGAACAGCCTT
TAAAAATTATAAGTGTAAAGATACAGTAGAAAGTGTGCGTGCAGTTTGGTGAAGAACCTTT
TGTAACAAATTGATATGA
ATGATTGCTTTGTGACTAGGCATGCCAAATATGTATAATTGGGACTGTATCTAAGTTTTC
TCAAGGTGACACTCTACTA
TTTTCTGGGGCCCATGGAAGGAGGTGGTATAATCTTTTAAACACTGGTGTACATCTACCTGTC
ACTTTGGAGACCTTGGTGA
TGTCATGGGTCCAAAAGATAAACCTTTTATTGCCCTGAATTTCCAGGGCAATTAGGAAA
AAATGTAACTTTGCCACAA
CTCCAGTTTGTGAATATGATGGAACATTATATCAGGCTATAAGAAAGTACTTGCAACAAT
TGATTTCTTCAATCATTT
AACACAAGCAATATACCTTCACTGATGAGAGAATTGAATGGAGAGACCTGATGGCATGC
TTCGGGATCATATTAATAT
TGTTATTCTTAAAGATATTGATTTTGAATTTGGCTGAGAATCCTTGTAAAGTAGGGCTC
CAGGCAGCAAAACATAGAAG
GTGCCTGGGGTTCAGGTGTGGGTTTACACTCACATGCAAGGTGTCTCTCACAGAATGCC
AACATTTCTTACATCAATA
AAGGCCGTGACATGGCAATTGTTATGGTGCAGAAAGTGTGACACTCTCACAGGACAAA
ATACTGTCAAAATTACCGG
GAAAGGTGGCCATAGTGTGTTCTTCAATCAAAATGCTGTCTATGGGAAAGAATGTTTCACTCAACT
GGCCTCCAAGCCAGTGCAC
CACATCTGGATAAGGTAATGGTATCTCTGAGTTAGAAAACGAGAAAGTTTATGATGACGG
TGCACCTGAATGTGGCATT

ACTTGTGTGGTTTAAAAAATCAGGTGAATGGGTATGGGTATAATCAATGGGAAC TGGGTG
 TCCTAAATTGCTCTGTGTGTG
 ACTGCTGCTCTTTTCTCTTATCTCTGTGAGCATCTTGTGTCC TGTGTAGAAAGCATAAAAA
 TCATAAAATCCACCTTAACA
 ATCTTCACATCATGTATCGATTTTCAAACAC TTTATCATTTAGAACTTAAC TTGGCACTAC
 TATCTGATAACTGACTTTC
 ATTTT TATTTT TATATGGATTAATTACTAAAAAAATACTCTCTCGTGCCGAATTCGATAT
 CAAGCTTATCGATACCGTC
 GACCTCGAGGGGGGGCCGGTACCCGGGATCC TCGCAATCCC TAGGAGGATTAGGCAAGGG
 CTTGAGCTCAGCTCTTTGT
 GAGGGACAGAAATACAATCAGGGGCAGTATATGAATACTCCATGGAGAAACCCAGATCTAC
 GTATGATCAGCCTCGACTG
 TGCCTTCTAGTGTGCCAGCCATCTGTGTGTTGCCCCC TCCCCGTCGCTTCC TTGACCCCTGGA
 AGGTGCCACTCCCCTGTCT
 CTTTCTTAATAAAATGAGGAAATGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGG
 GGGGTGGGTGGGGCAGGA
 CAGCAAGGGGGAGGATTGGAAGACAATAGCAGGCATGCTGGGGATCGGGTGGGCTCTATG
 GCTTCTGAGGCGGAAAGAA
 CCAGCTGGGGCTCGACAGCTCGACTCTAGAA TTGCTTCTCGCTCACTGACTCGCTCGCGCT
 CGGTCTGTTCCGCTGCGGGC
 AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA
 GGAAGAACAATGTGAGCAA
 AAGGCCAGCAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTT TTTCCATAGGCT
 CCGCCCCCTGACGAGCAT
 CACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGACTATAAAGATACCAGG
 CGTTTCCCCCTGGAAGCTC
 CCTCGTGCCTCTCCTGT TCCGACCTGCGCGTTACCGGATACCTGTCCGCC TTTCTCCCT
 TCGGGAAGCGTGGCGCTTT
 CTC AATGCTCACGCTGTGATATCTAGTTCCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTG
 TGTGCACGAACCCCCGTT
 CAGCCCAGCCGCTGCGCCTTATCCGGTAAC TATCGCTTTGAGTCCAACCCGGTAAGACACG
 ACTTATCGCCACTGGCAGC
 AGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAG
 TGGTGGCCTAACTACGGCT
 ACAC TAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAG
 AGTTGGTAGCTCTTGATCC
 GGCAAAACAAACACCGCTGGTAGCGGTGTT TTTTGTGTTGCAAGCAGCAGATTACGCGCA
 GAAAAAAGGATCTCAAGA
 AGATCCTTTGATCTTTTCTACGGGTCTGACGCTCAGTGAACGAAAACTACGTTAAGGG
 ATTTTGGTCAATGAGATTAT
 CAAAAAGGATCTTCACCTAGATCCTTTTAAAT TAAAAATGAAGTTT TAAATCAATCTAAAG
 TATATATGAGTAAACTTGG
 TCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTC TATTTCTGTT
 CATCCATAGTTGCCGTGACTC

SEQ ID NO:4: pWRG/SEO-S

GGGGGGGGGGGCGCTGAGGTCTGCCCTCGTGAAGAAGGTGTTGCTGACTCATACCAGGCCT
 GAATCGCCCCATCATCCAG
 CCAGAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGATT
 TTGAAC TTTTCTTTTGCCA
 CGGAACGGTCTGCGTTGTGCGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTCTG
 ATTTATTTCAACAAAGCCGC

CGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGTACAAACCAATTAACCAATTCTGATTA
GAAAAACTCATCGAGCATC
AAATGAAACTGCAATTTATTTTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTT
TCTGTAATGAAGGAGAAAA
CTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGT
CCAACATCAATACAACCTA
TTAATTTCCCCTCGTCAAAAAAAGGTATCAAGTGAGAAATCACCATGAGTGACGACTGA
ATCCGGTGAGAAATGGCAAA
AGCTTATGCATTCTTTCCAGACTTGTTCACAGGCCAGCCATTACGCTCGTCAATCAAAAT
CACTCGCATCAACCAACC
GTTATTCTATTGCTGATTGGCCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAGGACAA
TTACAAAACAGGAATCGAAT
GCAACCGCGCAGGAACACTGCCAGCGCATCAACAATATTTTACCCTGAATCAGGATATTC
TTCTAATACCTGGAATGCT
GTTTTTCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCT
TGATGGTCGGAAGAGGCAT
AAATTCGGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTTGGCAACGCTACCT
TTGCCATGTTTCAGAAACA
ACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTGCGACCTGATTGCCCGACATT
ATCGCGAGCCCCATTTATAC
CCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCCTCGAGCAAGACGTTTCCCGTT
GAATATGGCTCATAACACC
CCTTGTAATTACTGTTTATGTAAAGCAGACAGTTTATTTGTTTCATGATGATATATTTTTATCT
TGTGCAATGTAAATCAGA
GATTTTGAGACACAACGTTGGCTTCCCCCCCCCCCCCGGCATGCCTGCAGGTCGACATAAAAT
CAATATTGGCTATTGGCCA
TTGCATACGTTGTATCTATCATATAATATGTACATTTATATTGGCTCATGTCCAATATGAC
CGCCATGTTGACATTTGATTT
ATTGACTAGTTTATTAATAGTAATCAATTACGGGGTCAATTAGTTCATAGCCCATATATGGAG
TTCCGCGTTACATAACTTA
CGGTAAATGGCCCGCTCGTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACG
TATGTTCCCATAGTAACGC
CAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTACGGTAAAC'TGCCCACTTGGC
AGTACATCAAGTGATCAT
ATGCCAAGTCGGGCCCTTATTGACGTCAATGACGGTAAATGGCCCGCTGGCATTTATGCC
CAGTACATGACCTTACGGG
ACTTTCCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTT
TTGGCAGTACACCAATGGG
CGTGGATAGCGGTTTGACTCACGGGGATTTCGAAGTCTCCACCCCATTTGACGTCAATGGGA
GTTTGTTTTGGCACCAGAA
TCAACGGGACTTTTCCAAAATGTCGTAAATAACCCCGCCCGTTGACGCAATGGGCGGTAGG
CGTGTACGGTGGGAGGCTT
ATAAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTT
TGACCTCCATAGAAGACAC
CGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCAATTGGAACGCGGATTTCCCGTGCCA
AGAGTGACGTAAATACCGC
CTATAGACTCTATAGGCACACCCCTTTGGCTCTTATGCATGCTATACTGTTTTTGGCTTGG
GGCCTATACACCCCGCTC
CTTATGCTATAGGTGATGGTATAGCTTAGCCTATAGGTGTGGGTATTGACCATTATTGAC
CACTCCCTATTTGGTGAG
ATACTTTCCATTACTAAATCCATAACATGGCTCTTTGCCACAACATCTCTATTGGCTATAT
GCCAATACTCTGTCTCTCA
GAGACTGACAGGACTCTGTATTTTACAGGATGGGGTCCCATTTATTATTTACAAAATTCA
CATATACAACACGCGCTC

CCCCGTGCCCGCAGTTTTTATTAACATAGCGTGGGATCTCCACGCGAATCTCGGGTACGT
GTTCCCGGACATGGGCTCTT
CTCCGGTAGCGGCGGAGCTTCCACATCCGAGCCCTGGTCCCATGCGCTCCAGCGGCTCATGG
TCGCTCGGCAGCTCCTTGC
TCTTAACAGTGGAGGCCAGACTTAGGCACAGCACAATGCCACCACCACAGTGTGCCGCA
CAAGGCCGTGGCGGTAGGG
TATGTGCTGAAAAATGAGCTCGGAGATTGGGCTCGCACCGTGACGCAGATGGAAGACTTAA
GGCAGCGGCAAGAAGAAT
GCAGGCAGCTGAGTTGTTGTATTC'TGATAAGAGTCAGAGGTAAC'TCCCGTTCGGGTGCTGT
TAACGGTGGAGGGCAGTGT
AGTCTGAGCAGTACTCGTTGCTGCCGCGCGCCACCAGACATAATAGCTGACAGACTAAC
AGACTGTTTCCTTCCATGG
GTC'TTTTCTGCAGTCACCGTCCAAGCTTTCGGGCCAATTCGGCACGAGAGAGTAGTAGACTC
CCTAAAGAGCTACTACACT
AACAGAAAAAATGGCAACTATGGAAGAAATCCAGAGAGAAATCAGTGCTCACGAGGGGCAG
CTTGATAGCACGCCAGA
AGGTCAAGGATGCAGAAAAGCAGTATGAGAAGGATCCTGATGACTTAAACAAGAGGGCAGT
GCATGATCGGGAGAGTGTCT
GCAGCTTCAATACAATCAAAAATTTGATGAATTGAAGCGCCAAC'TTCCGCACAGATTGCAGC
AGGGAAGAACATTCGGGGA
GGACCGGGATCTTACAGGGGTAGAGCCAGGTGATCATCTTAAAGAAAGATCAGCACTAAGC
TACGGGAATACACTGGACC
TGAATAGTCTTGACATTGATGAACCTACAGGACAGACAGCTGATTGGCTGACCATAATTGT
CTATCTGACATCATTCGTG
GTCCCGATCATCTTGAAGGCAGTGTACATGTTAAACAACAGAGGTAGGCAGACTTCAAAAG
ACAACAAGGGGATGAGGAT
CAGATTCAAGGATGACAGCTCATATGAGGATGTCAATGGAATCAGAAAGCCAAACATCTG
TATGTGTCAATGCCAAACG
CCCAATCCAGCATGAAGGCTGAAGAGATAACACCAGGAAGATTCGCAC'TGCAGTATGTGG
ACTATATCTTCGCACAGATA
AAGGCAAGGAATATGGTAAGCCCTGTCAATGAGTGTAGTTGGGTTC'TTGGCAGCTGGCAAAAG
ACTGGACATCGAGAATTGA
AGAATGGCTCGGTGCACCTGCAAAATTCATGGCGGAGTCTCTTAT'TGCCGGGAGTTTATCT
GGGAATCCTGTGAATCGTG
ACTATATCAGACAGAGACAAGGTGCACTTGCAGGGATGGAGCCAAAGGAATTTCAAGCCCT
CAGGCAACATTCAAAGGAT
GCTGGATGTACACTAGTTGAACATATTGAGTCACCATCATCAATATGGGTGTTTGTCTGGGG
CCCCGTAGAGTGTCCACC
AACATGCTTGT'TTGTTCGGAGGGATGGCTGAATTAGGTGCTCTCTTTTCTATATTCAGGAT
ATGAGGAACACAATCATGG
CTTCAAAAAC'TGTGGGCAAGCTGATGAAAAGCTTCGAAAAGAAATCATCAT'TCTATCAATC
ATACCTCAGACGCACACAA
TCAATGGGAATACAAC'TGGACAGAGGATAATGTGTTATGTTTATGGTTGCTGGGGAAGG
AGGCAGTGGACAAC'TTCA
TCTCGGTGATGACATGGATCCAGAGCTTCGTAGCC'TGGCTCAGATCTTGATTGACCAGAAA
GTGAAGGAAATCTCAAAAC
AGGAACTTATGAAATATATAGTACATAAATATATAATCAATACTAACTATAGGTTAAGAAA
TACTAATCATTTAGTTAATA
AGAAATAGATTTATTTGAATAATCATATTAAATAATTAGTAAAGTTAACTAGTATTTAGTT
AAGTTAGCTAATTTGATTTA
TATGATTGTCACAATTAATGTAATCATAAGCACAATCACTGCCATGTATAATCACGGGTA
TACGGGTGGTTTTCATATG
GGGAACAGGGTGGGCTTAGGGCCAGGTCACCTTAAGTGACCTTTTTTTGTATATATGGATG
TAGATTTCAATTTGATCGAA

TACTAATCCTACTGTCTCTTTTCTTTTCTCTTCTCTTTTACTAACAACTACCTCGT
 GCCGAATTTGGCCGCGGATC
 CTCGCAATCCCTAGGAGGATTAGGCAAGGGCTTGAGCTCACGCTCTTGTGAGGGACAGAAA
 TACAATCAGGGGCAGTATA
 TGAATACTCCATGGAGAAACCCAGATCTACGTATGATCAGCCTCGACTGTGCCTTCTAGTT
 GCCAGCCATCTGTTGTTG
 CCCCTCCCCCGTGCCTTCCCTTGACCTTGAAGGTGCCACTCCCACTGTCTTTTCTTAATAA
 AATAGGAAATTTGCATCGC
 ATTTCTCTGAGTAGGTGTCTATTCTATTTCTGGGGGTGGGGTGGGGCAGGACAGCAAGGGGA
 GGATTGGGAAGACAATAGC
 AGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCT
 CGACAGCTCGACTCTAGAA
 TTGCTTCTCTCGCTCACTGACTCGCTGCGCTCGGTCTGCTGGCTGCGCGAGCGGTATCAGC
 TCACTCAAAGGCGGTAAATA
 CGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGCCAGCAAA
 AGGCCAGGAACCGTAAAAA
 GGCCGCGTTGCTGGCGTTTTCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGA
 CGCTCAAGTCAAGAGGTGGC
 GAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCCTC
 TCTGTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTGCTGGCTGCGCGAGCGGTATCAGC
 CTTACCGGATACCTGTCCGCTTCTCTCCCTTCGGGAAGCGTGGCGCTTCTCAATGCTCAC
 GCTGTAGTATCTCAGTTC
 GGTGTAGGTCTGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTAGCCCGACCGC
 TGCCTTTATCCGGTAACT
 ATCGTCTTTGAGTCCAACCCGGTAAGACAGCAATTATCGCCACTGGCAGCAGCCACTGGTAA
 CAGGATTAGCAGAGCGAGG
 TATGTAGGCGGTGCTACAGAGTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGA
 CAGTATTTGGTATCTGCGC
 TCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAACAAAC
 ACCGCTGGTAGCGGTGGTT
 TTTTGTGTTGAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGAT
 CTTTTCACGGGTCTGAC
 GCTCAGTGAACGAAACTCAGTCTAAGGATTTTGGTATGAGATTATCAAAAGGATCT
 TCACCTAGATCCTTTTAA
 TTAAAAATGAAGTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTAC
 CAATGCTTAATCAGTGAGG
 CACCTATCTCAGCGATCTGTCTATTTCGTTTATCCATAGTTGCCTGACTC

SEQ ID NO: 1: Sapporo rat (SR11) seoul M

TAGTAGTAGACTCCGCAAGAAACAGCAGTTAAAGAACAATAGGATCATGTGGAGTTTGCTA
 TTACTGGCCGCTTTAGTTG
 GCCAAGGCTTTGCATTAATAAATGTATTTGACATGAGAATTCAAGTTGCCCACTCAGTCAA
 CTTTGGGGAACAAGTGTG
 TCAGGCTATACAGAAATTTCCCCCACTCTCATTTACAGGAGGCAGAACAGCTAGTGCCAGAGA
 GCTCATGCAACATGGACAA
 CCACCAGTCACTCTCAACAATAAATAAATTAACCAAGGTATATGGCGGAAAAAAGCAAAT
 CAGGAATCAGCAAAACCGA
 ATTCATTTGAAGTTGTGGAAGTGAAGTCAGCTTAAAGGGTTGTGTATGTTAAAGCATAG
 AATGGTTGAAGAATCATAT
 AGAAATAGGAGATCAGTAATCTGTTATGATCTAGCCTGTAATAGTACATTCTGTAAACCA
 CTGTTTATATGATTTGCTC
 TATACATGCTTGCAACATGATGAAAAGCTGTTTGAATTGGCCTTGGCCCCCTACAGAATCCAG
 GTTGTCTATGAAAGGACAT

ACTGCAC TACGGGTATAT TGACAGAAGGAAAATGCTTTGTCCCTGACAAGGCTGTTGTCA
TGCATTGAAAAGAGGCATG
TATGCTATAGCAAGCATAGAGACAATCTGCTTTTTTATTATCATCAGAAAGGGAATACATATA
AGATAGTGACTGCCATTAC
ATCAGCAATGGGCTCCAAATGTAATAATACAGATACTAAAGTTCAAGGATATTATATCTGT
ATTATTGGTGGAACTCCG
CCCCGTGATATGCCCTGTCTGTGAAGACTTCAGAGCAATGGAGGTTTTTCTGGGATTAT
TACATCACCAATGGAGAA
GACCATGACCTACCCGGCGAAGAAATCGCAACGTACCAGATTTCAAGGCAGATAGAGGCAA
AAATCCCTCATACAGTGAG
CTCCAAAACCTTAAATTTGACTGCTTTTGCAGGTATTTCCATCATACTCATCAACTAGTATA
TTGGCTGCTTCAGAAGATG
GTCGTTTCATATTTAGTCTCTGGTTTATTTCCTAACCTAAATCAGTCAGTCTGTGACAACAA
TGCCTCCCTTTAACTCTGG
AGGGGCTTAATTGATTTAACGGGATACTATGAGGCAGTCCACCTTGAATGTGTCTGTG
TCTTATCAGGACCAGGTGC
TTCATGTGAGGCTTTTCAGAAGGAGGTATTTCAATATTACTTCTCCAATGTGTCTGGTG
TCTAAGCAAAAATAGATTTA
GAGCAGCTGAGCAGCAGATTAGCTTTGTCTGCCAAAGAGTTGATATGGATATTATAGTGTA
CTGTAATGGTCAGAAAAA
ACAACTCTAAACAAAACATTAGTTATAGGCCAATGTATTTATACTATTACAAGTCTCTTTT
CACTGTTACCGGGGTG
CCATTCTATTGCTATTGAGTTGTGTGTTCCAGGTTTCATGGCTGGGCCACAGTGCCTTT
TTGATTACATTCTGCTCG
GCTGGGTATTGATTCTCTGCATGTACATTAGCTATTCTTTTAGTCTTAAGTCTTTGCAAA
TATCTTTCATACAAGCAAT
CAAGAGAACCGATTCAAAGCCATTCTACGAAAAATAAGGAGGAGTTGAAAAACAAAGG
GTTCCATGGTTGTGAGAT
CTGTAAGTATGAGTGTGAACATTAAGGAATTGAAGGCACATAACCTATCATGTGTTCAA
GGAGAGTGCCCATATTGCT
TTACCCACTGTGAACCGACAGAACTGCAATTCAGGCACATTACAAGTTTGTCAAGCCAC
CCACCGATTTCAGAGAAGAT
TTAAAAAAGACTGTAACTCTCTCAAATATTGGGCCAGGCTGTTACCGAACACTAACTCTTT
TTAGGTATAAAGTAGGTG
TTATATTCTGACAAATGTGGACTCTTCTCTCATTAATTGAATCCATCTCTGGGCAGCAAGT
GCAGCAGAAATCCCCCTTG
TCCCTCTCTGGACAGATAATGCTCATGGCGTTGGGAGTGTCTCATGCATACGGATCTTGA
ATTAGACTTCTCTTTTGCCA
TCCAGTTCTAAGTACACATACAAAAGACATCTCACAACCCAGTTAATGACCAACAGAGTG
TCTCATTTGCATATAGAAAT
TGAAAGTCAAGGCATTGGTGCTGCTGTTTCATCATCTTGACATTGGTATGATGCAAGATTG
AATCTAAAAACCTCATTTT
ATTGTTATGGTGCCTGCACAAAATATCAATACCCATGGCACACTGCAAAATGCCATTTTGA
GAAAGATTATGAGTATGAA
AATAGCTGGGCTTGCAACCCCTCCAGATTGCCAGGGGTTGGTACAGGTTGTACTGCTTGTG
GATTATATCTAGATCAATT
GAAGCCGGTAGGAACAGCCTTTAAAAATTATAAGTGTAAGATACAGTAGAAAAGTGTGCGTG
CAGTTTGGTGAAGAACACC
TTTGTAAAACAAATTGATATGAATGATGCTTTGTGACTAGGCATGCCAAAAATATGTATAAT
TGGGACTGTATCTAAGTTT
TCTCAAGGTGACACTCTACTATTCTGGGGCCCATGGAAGGAGGTGGTATAATCTTTAAAC
ACTGGTGTACATCTACCTG
TCACTTTGGAGACCTGTGTGATGTATGGGTCCAAAAGATAAACCATTTATTTTGGCCCTGAA
TTCCAGGGCAATTTAGGA

AAAAATGTAACCTTTGCCACAACCTCCAGTTTGTGAATATGATGGAAACATTATATCAGGCTA
 TAAGAAAGTACTTGCAACA
 ATTGATTCTTTCCAATCATTTAACACAAGCAATATACACTTCAGTGATGAGAGAATTGAAT
 GGAGAGACCCGTGATGGCAT
 GCTTCGGGATCATATTAAATATTGTTATTTCTAAAGATATTGATTTTGAATAATTGGCTGAG
 AATCCTTGTAAAGTAGGGC
 TCCAGGCAGCAACATAGAAAGGTGCTCGGGTTTCAGGTGTGCGGGTTTACACTCAGATGCAA
 GGTGCTCTCTACAGAATGC
 CCAACATTTCTTACATCAATAAAGGCCTGTGACATGGCAATTTGTTATGGTGCAGAAAGTG
 TGACACTCTCAGCAGGACA
 AAATACTGTCAAAATTACCGGAAAGGTGGCCATAGTGGTCTTCATTCAAATGCTGTTCAT
 GGGAAAGAAATGTTCAATCAA
 CTGGCCTCCAAGCCAGTGACACACATCTGGATAAGGTAATGATCTCTGAGTTAGAAAA
 CGAGAAAGTTTATGATGAC
 GGTGCACCTGAATGTGGCATTAATTGTTGGTTTAAAAAATCAGGTGAATGGGTTATGGGTA
 TAATCAATGGGAACGGGT
 TGTCTTAATTGTCTTGTGTGTACTGCTGCTCTTTTCTCTTATCCTGTTGAGCATCTTGTGT
 CCTGTTAGAAAGCATAAAA
 AATCATAAATCCCACCTAACAACTCTTCACATCATGTATCGATTTTCAAACACTTTATCATT
 TAGAACTTAACTTGGCACT
 ACTATCTGATAACTGACTTTTCATTTTATTTTATATGAGTAATTAATAAAAAAATACT
 CTCTTCTATCTCCCAATCT
 TTTATTGATTACCGGGGTGCTGTCTTGACATCTGGCGGCGTCTACTACTA

SEQ ID NO:2: Sapporo rat virus (strain SR-11),
 seoul S

TAGTAGTAGACTCCCTAAAGAGCTACTACACTAACAAAGAAAAATGGCAACTATGGAAGAAA
 TCCAGAGAGAAATCAGTGC
 TCACGAGGGGCAGCTTGTGATAGCAGCCAGAAGGTCAAGGATGCAGAAAAGCAGTATGAG
 AAGGATCCTGATGACTTAA
 ACAAGAGGGCACTGCATGATCGGGAGAGTGTGCGAGCTTCAATACAATCAAAATTTGATGA
 ATTTGAAGGCCAATCTGGC
 GACAGATTGCAGCAGGGAAGAACATCCGGGCAGGACCGGGATCCTACAGGGGTAGAGCCAG
 GTGATCATCTTAAGGAAAG
 ATCAGCACTAAGCTACGGGAATACACTGGACCTGAATAGTCTTGACATTGATGAACCTACA
 GGACAGACAGCTGATTGGC
 TGACCATAAATTGTCTATCTGACATCATTCGTGGTCCCGATCATCTTGAAGGCAGCTGTACAT
 GTTAACAACACGAGGTAGG
 CAGACTTCAAGGACAACAAGGGGATGAGGATCAGATTCAAGGATGACAGCTCATATGAGG
 ATGTCAATGGAAATCAGAAA
 GCCCAAACATCTGTATGTGTCAATGCCAAACGCCAATCCAGCATGAAGGCTGAAGAGATA
 ACACCGGAAGATTCCGCA
 CTGCAGTATGTGGACTATATCCTGCACAGATAAAGGCAAGGAATATGGTAAGCCCTGTTCAT
 GAGTGTAGTTGGGTTCTTG
 GCACTGGCAAAAGACTGGACATCGAGAATTGAAGAATGGCTCGGTGCACCCCTGCAAAATCA
 TGGCGGAGTCTCTTATTGC
 CGGGAGTTTATCTGGGAATCCTGTGAATCGTGACTATATCAGACAGAGACAAGGTGCACCTT
 GCAGGGATGGAGCCAAAGG
 AATTTCAAGCCCTCAGGCAACATTCAAGGATGCTGGATGTACACTAGTTGAACATATTGA
 GTCACCATCATCAATATGG
 GTGTTTGTCTGGGGCCCTGATAGGTGTCCACCAACATGCTTGTGTTGCGAGGGATGGCTG
 AATTAGGTGCCTTCTTTTC

TATACTTCAGGATATGAGGAACACAATCATGGCTTCAAAAAC'TGTGGGCACAGCTGATGAA
AAGCTTCGAAAGAAATCAT
CATTCTATCAATCATACCTCAGACGCACACAATCAATGGGAATACAAC'TGGACCAGAGGAT
AATTGTTATGTTTATGGTT
GCCTGGGGAAAGGAGGCAGTGGACAAC'TTCATCTCGGTGATGACATGGATCCAGAGCTTC
GTAGCCTGGCTCAGATC'TT
GATTGACCAGAAAGTGAAGGAAATCTCAAACCAGGAACCTATGAAAT'TATAAGTACATAAA
TATATAATCAATACTAACT
ATAGGTTAAGAAATACTAATCAT'TAGTTAATAAGAATATAGAT'TTATTGAATAATCATATT
AAATAATTAGGTAAGTTAA
CTAGTATT'TAGTTAAGTTAGCTAATTGATTTATATGATTGTCACAATTAAATGTAATCATA
AGCACAATCACTGCCATGT
ATAATCACGGGTATACGGGTGGT'TTTCATATGGGGAACAGGGTGGGCTTAGGGCCAGGTCAC
CCTTAAGTGACCTTTTTTTT
GTATATATGGATGTAGATTTCAATTGATCGAATACTAA'TCCTACTGTCC'TCTTTTCTTTTC
CTTTCTCCTTCTTTACTAA
CAACAACAACTACCTCAGACCTTAATATATACTACTTTTATTAAAGTTGTTAAGTTGTGTCT
TTTTGGGGAGTAAGGGAGT
CTACTACTA

